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Mechanisms of post-transcriptional regulation of genes involved in FTDP-17

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Declaration of authorship

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Signed

To all patients with Frontotemporal Dementia

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Abstract

MicroRNAs (miRNAs) are small non coding RNAs of 18-25 nt, capable of regulating mRNA translation and gene expression at post-transcriptional level. Alteration of miRNAs expression is often associated with human diseases, such as cancers and neurodegenerative pathologies. The main objective of this study is an analysis of the post-transcriptional regulation played by miRNAs of two important genes, *MAPT* and *GRN*, involved in Frontotemporal Dementia with Parkinsonism linked to chromosome 17 (FTDP-17). This is one of the major degenerative dementia syndromes, characterized by atrophy of the prefrontal and anterior temporal lobes. Several studies identified 44 pathogenic mutations in *MAPT*, which encodes for microtubule associated tau protein. In the brain tau has important functions in the assembly and stability of microtubules, that are fundamental for neuronal integrity and function. Disruption of tau role due to tau aggregations cause devastating effects that trigger neurodegeneration. To date 69 different mutations were found in *GRN* in the presence of FTD. *GRN* encodes a secreted precursor protein called progranulin that is expressed in neurons, microglia and represents an important growth factor involved in the regulation of multiple processes. The study identified miRNAs that can bind to GRN and MAPT mRNAs, affecting the translation efficiency of these transcripts, with a consequently reduction of the protein production. The project combined standard bioinformatic tools with a novel capture affinity assay, called miR-CATCH, in order to identify the best putative miRNAs. The assay was performed with specific biotinylated anti-sense oligonucleotides for the pull-down of GRN and MAPT mRNAs in different neuroblastoma cell lines. The binding of selected miRNAs on the 3'UTR regions of these genes, was validated using over-expressing vectors and reporter constructs to perform luciferase assays. Regulation of selected miRNAs over-expression on progranulin and tau proteins was investigated in a neuroblastoma cell line, using western blot. This effect was further analyzed with Real Time PCR reactions and polysomal analysis to understand if the selected miRNAs control progranulin and tau expression through a mechanism of translational repression or transcripts degradation. Two of the analyzed candidates, miR-659-3p and miR-608 were found important for the regulation of both genes, *GRN* and *MAPT* implicated in the disease, with a subsequent control on the endogenous progranulin and tau production in neuroblastoma cell line. Whereas miR-939-5p and miR-615-5p are only

involved in the regulation of GRN expression. In addition we identified several SNPs located on miRNAs binding sites that are predicted to increase or decrease miRNAs binding. Since neurological disorders are strongly influenced by common genetic variability, SNPs or variations that overlapped miRNA-binding sites, could represent potential and important risks factors for generation of FTD.

List of Abbreviations

FTD	Frontotemporal dementia
FTLD	Frontotemporal lobar degeneration
MAPT	Microtubule associated protein tau gene
VCP	Valosin-containing protein
CHMP2B	Charged multivesicular body protein 2B
C9ORF72	Chromosome 9 open reading frame 72
TARDBP	TAR DNA binding protein
FUS	Fused in sarcoma
ALS	Amyotrophic lateral sclerosis
i-CLIP	Individual-nucleotide resolution cross-linking and immunoprecipitation
RNA	Ribonucleic acid
miRNA	microRNA
IBMPFD	Inclusion Body Myopathy with Paget's disease of the bone and frontotemporal dementia
ESCRT-III	Endosomal sorting complex required for transport-III
VPS4A	Vacuolar protein sorting 4
RAN	Repeat-associated non-ATG translation
ER	Endoplasmic reticulum
SORT1	Sortilin1
TNFR	Tumor necrosis factor receptor
CNS	Central nervous system
CSF	Cerebrospinal fluid
NFT	Neurofibrillary tangles
RISC	RNA-induced silencing complex
UTR	Untranslated region
RT-PCR	Reverse-transcriptase polymerase chain reaction
cDNA	Complementary DNA
luc	Luciferase

FBS	Fetal bovine serum
EMEM	Eagle's minimum essential medium
DMEM	Dulbecco's modified eagle's medium
ELISA	Enzyme-linked immunosorbent assay
miR-CATCH	MiRNA Capture Affinity Technology
RIPA	Radio immuno precipitation assay
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
HPRT1	Hypoxanthine phosphoribosyltransferase
SDHA	Succinate dehydrogenase complex subunit A
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
qRT-PCR	Quantitative Real Time PCR
sRNU	Small nuclear RNA
AD	Alzheimer's disease
ROS	Reactive oxygen species
HIF	Hypoxia inducible factor
HREs	Hypoxia responsive elements
SNP	Single nucleotide polymorphism

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CHAPTER THREE: IDENTIFICATION OF miRNAs INVOLVED IN THE POST-TRANSCRIPTIONAL REGULATION OF *GRN* EXPRESSION

CHAPTER ONE: INTRODUCTION

1.1 Frontotemporal Dementia (FTD): General Introduction

Frontotemporal dementia (FTD) is a neurodegenerative disease, characterized by atrophy of the frontal and anterior temporal lobe that determines behavior and personality changes with a gradual deterioration of languages skills. All the pathological manifestations associated with FTD are collectively termed frontotemporal lobar degeneration (FTLD) (Nicholson A.M. *et al.* 2011). After Alzheimer's disease, the second most common cause of early-onset dementia is Frontotemporal lobar degeneration (FTLD) (Graff-Radford N.R and Woodruff B.K. 2007). Indeed FTLD affects mainly individuals under the age of 65 (Nicholson A.M. *et al.* 2011). The 30-50% of FTLD cases have a familial component (Lashley T. *et al.* 2011), although sporadic forms of FTLD are also known. In 10-23% of cases the disorder segregates with an autosomal dominant inheritance pattern (Goldman J.S. *et al.* 2005 and 2007). Molecular genetic studies have identified so far seven genes that cause FTLD in presence of mutations: the microtubule associated protein tau gene (MAPT), progranulin gene (GRN), the gene encoding valosin-containing protein (VCP), the charged multivesicular body protein 2B (CHMP2B), abnormal expansion of a hexanucleotide repeat in C9orf72, TARDBP and FUS genes (Cruts M. *et al.* 2012). The last three genes can also cause a clinical picture of amyotrophic lateral sclerosis (ALS), or a combination of FTD and ALS (ALS/FTD) (Pearson J.P. *et al.* 2011; Neumann N. *et al.* 2006, Yan *et al.* 2010). Frontotemporal dementia appears to be a complex pathology caused by an heterogeneous group of different genes that lead to different neuropathological characteristics (Cairns NJ, *et al.* 2007). Most cases of FTLD are classified into different categories according to the abnormal intracellular accumulations of specific protein that are found in patients (**Table 1**). The first identified subgroup is characterized by aggregation of hyperphosphorylated tau protein in neuron and glia (FTLD-tau), a second group is characterized by neuronal inclusion originally positive for ubiquitin (FTLD-U), afterwards called FTLD-TDP since in most cases the ubiquitinated protein found in the pathological inclusions is the transactive response DNA

binding protein (TDP-43) (Arai T *et al.* 2006). Subsequently a third subgroup was identified with inclusions immunoreactive for FUS termed (FTLD-FUS) (Neumann N *et al.* 2009).

Molecular class	Pathological subtype*	Associated genes	Clinical phenotypes				
			bvFTD	nfvPPA	svPPA	Park	MND
FTLD-tau		• *MAPT*	+	(+)	(+)	+	ALS, PLS
	• PiD		+	+	(+)		
	• CBD		+	+		+	PLS
	• PSP		+	+		+	PLS
	• AGD		+				
	• NFT-dementia		+				
	• MSTD		+			+	PLS
FTLD-TDP		• (*TARDBP*)	(+)			+	ALS
	• Type A	• *GRN*	+	+		+	
	• Type B	• *C9orf72*	+	+	(+)	+	ALS
	• Type C		+		+		
	• Type D	• *VCP*	+			(+)	ALS
FTLD-FUS		• (*FUS*)	(+)				ALS
	• aFTLD-U		+				
	• NIFID		+			+	PLS
	• BIBD		+			+	ALS
FTLD-UPS	• FTD-3	• *CHMP2B*	+			(+)	(ALS)

Table 1. Molecular classification of FTLD with the genes involved in the pathology and the clinical phenotypes. * Pathological subtype indicates the pattern of pathology (Adapted from Riedl *et al.* 2014).

1.1.1 The Molecular Genetics of FTLD

A large proportion of FTD patients have a family history, the first two genes that were identified associated with FTD were *MAPT* and *GRN*, located on chromosome 17 (Baker *et al.* 2006). These genes will be further discussed in the following chapters (Chapter three and Chapter four). TDP-43, encoded by *TARDBP* (TAR DNA Binding Protein) gene is a ubiquitous protein of 43 KDa and 414 amino acids (Wang *et al.* 2008; Neumann *et al.* 2006). This protein is mainly localized in the nucleus, however a cytosolic expression was observed in large motor neurons, with a role in mRNA transport and dendritic branching (Ayala *et al.* 2005, Wang *et al*. 2008; Barmada *et al*., 2010; Kabashi *et al.* 2010). After oxidative stress, TDP-43 can also accumulate in cytoplasmic stress granules, which usually represent a temporarily storage of non-translating mRNAs, RNA-binding proteins, small ribosome subunits and translation initiation factors (Colombrita *et al.* 2009; Buchan and Parker, 2009). Different works showed that TDP-43 can regulate more than 6,000 RNA targets, through a binding to introns, 3'UTRs and non-coding RNAs (Polymenidou *et al.* 2011, Tollervey *et al*.

2011). TDP-43 is not only involved in mRNA transport, stability and maturation but increasing evidences reveals its role on splicing process, influencing the splice site selection through a binding on exon-intron junctions and intronic regions (Tollervey *et al*. 2011).

TDP-43 was also found involved in miRNA biogenesis, since it is able to associate with the Drosha microprocessor and directly interact with Drosha at different levels, influencing miRNAs production (Di Carlo *et al.* 2013). i-CLIP experiments demonstrated that TDP-43 could interact and bind to long non coding RNAs (lncRNAs) such as the nuclear-enriched autosomal transcript 1 (NEAT1) and the metastasis lung adenocarcinoma transcript 1 (MALAT1) (Tollervey *et al*. 2011). Even though the binding and regulation of non coding RNAs (ncRNAs) and miRNAs on TDP-43 expression need to be further investigate, TDP-43 can autoregulate its own expression through the activation of a cryptic polyA site and a nuclear retention mechanism (Avendaño-Vázquez *et al*. 2012). So far 34 different TDP-43 mutations have been found in different FTD or ALS/FTD families (Cruts *et al*. 2012), indicating that an altered role of TDP-43 can lead to neurodegeneration and frontotemporal dementia. Another RNA and DNA binding protein that present similar localization and functions is FUS, a multifunctional protein, often associated with TDP-43. Indeed FUS is actively implicated in mRNA transport, stability and translation, shuttling between the nucleus and the cytoplasm through a nuclear localization signal, as reported for TDP-43 (Iko *et al*. 2004). Different papers reported colocalization for FUS and TDP-43 in some nuclear complexes (Kim *et al*. 2010, Ling *et al*. 2010) or cytoplasmic aggregates of ALS/FTD affected neurons (Da Cruz and Cleveland, 2011). However, their colocalization seems to occur in specific situations and not all the aggregates found in post-mortem brain from FTLD patients contain both proteins (Neumann *et al.* 2009). Through the interaction of different splicing factors such as SRm160, PTB, serine/arginine proteins (SR proteins) and the direct binding to intronic or splice site sequences, FUS influences the splicing process (Meissner *et al*. 2003; Yang *et al*. 1998; Colombrita *et al.* 2012). It was reported that FUS could also affect the transcription process, through the binding of the C-terminal domain of RNA polymerase II, preventing the premature hyperphosphorylation of Ser2 in the C-terminal part of the RNA polymerase II (Schwartz *et al.* 2012). Similarly to TDP-43, FUS is involved in miRNA biogenesis, interacting with pri-miRNAs and helping the recruitment of Drosha for the correct production of miRNAs. The biogenesis of different miRNAs such as miR-9, miR-132 and miR-125b, that are found to be important for neuronal differentiation and

synaptogenesis, is controlled by FUS (Morlando *et al.* 2012). FUS can also bind ncRNAs such as CCND1, inducing an allosteric change of FUS and allowing the interaction between FUS and CBP/p300 that lead to the inhibition of histone acetyltransferase function of CBP/p300 (Wang X. *et al.* 2008). FUS was also found to interact with NEAT1 ncRNA, which assembles and organizes the core proteins of paraspeckles (Wang X. *et al*. 2008; Hoell *et al*. 2011; Lagier-Tourenne *et al.* 2012). Paraspeckles are present in almost all the cultured cells and in tissues with high levels of NEAT1 RNA, representing a nuclear retention of long hyperedited transcripts or a storage for the rapid release of RNAs during stress conditions (Prasanth *et al.* 2005; Chen and Carmichael, 2009). Reported mutations and aberrations localized mainly in the glycine rich region and C-terminal part of FUS are linked to the pathogenesis of FTD, ALS or ALS/FTD (Kwiatkowski *et al.* 2009; Vance *et al.* 2009; Lagier-Tourenne *et al.* 2010).

So far 18 different VCP mutations have been found in families with ALS/FTD, inclusion body myopathy and Paget disease (Cruts *et al.* 2012). A mutation identified on the VCP gene in a French family leaded to the Inclusion Body Myopathy with Paget's disease of the bone and frontotemporal dementia (IBMPFD), with a psychiatric onset of front temporal dementia and aggregation of TDP-43 (Jacquin *et al.* 2013).

VCP is one of the most abundant cytosolic protein in mammals and member of the AAA-ATPase gene superfamily (ATPase Associated with diverse cellular Activities) (Woodman, 2003; Wang *et al*. 2004). The protein is formed by 806 amino acids, with a N-terminal domain implicated in ubiquitin binding function and C-terminal domain with a strong ATPase activity (Wang *et al*. 2004). VCP is involved in the extraction of polyubiquitin chains from ubiquitinated proteins to facilitate the delivery to the proteasome (Meyer *et al.* 2012), especially in ER-associated degradation (Stolz *et al*. 2011). The first role that was associated with VCP was the regulation of the membrane dynamics, since VCP affects the reformation of the Golgi apparatus after mitosis and the ER network maintenance (Uchiyama and Kondo, 2005). Recently, evidences connect VCP with other membrane-mediate processes: endosomal trafficking and autophagy (Ramanathan and Ye, 2011; Ritz *et al*. 2011; Zehner *et al*. 2011). Another protein with a similar function in these two processes and implicated in the pathogenesis of frontotemporal dementia is the charged multivesicular body protein (CHMP2B).

CHMP2B is a 213 amino acids protein, component of the Endosomal Sorting Complex Required for Transport-III (ESCRT-III complex). This complex has an important role in the

endocytosed protein trafficking from endosome to lysosomes, acting at the sorting phase of protein and regulating their efficient lysosomal degradation (Urwin *et al*. 2010). Endosomal trafficking represents a fundamental process for the internalization, transport and secretion of signaling molecules or neuronal growth factors and an alteration of this mechanism could have heavy effects on communication between cells (Bronfman *et al*. 2007). In the cytosol, the C-terminal domain interacts with the N-terminal part, creating an autoinhibitory structure that require an activation done by VPS4, through a binding on the C-terminal domain of CHMP2B (Whitley *et al*. 2003; Shim *et al*. 2007). VPS4 is indeed a member of the AAA-ATPase family, with a role in catalyzing the dissociation of ESCRT complexes from endosomes and its activity is essential for the correct function of the endosomal sorting (Katzmann *et al*. 2002; Obita *et al*. 2007). CHMP2B is expressed in all the major parts of the brain, with an enhanced expression in the hippocampus, in the frontal, temporal lobes and in the cerebellum observed through an in situ hybridization of mouse brain (Skibinski *et al*. 2005). Mutations found in the CHMP2B gene are reported in frontotemporal dementia linked to chromosome 3 (FTD-3), an autosomal dominant dementia with an onset of 48-67 years, usually characterized by TDP-43 negative, ubiquitin positive inclusions (FTLD-U) (Urwin *et al.* 2010). Different missense mutations in the CHMP2B gene are connected with FTD-3 (Skibinski *et al*. 2005), with familial or sporadic cases of ALS (Urwin *et al*. 2010, Parkinson *et al*. 2006), familial frontotemporal lobar degeneration (FTLD) (Ghanim *et al*. 2010) or cortico-basal degeneration (CBD) (Van der Zee *et al*. 2008), however only few studies analyzed the pathogenic features of these mutations.

Recently a large expansion of the hexanucleotides non-coding repeat GGGGCC in the C9ORF72 was demonstrated to cause ALS and FTD (De Jesus-Hernandez *et al*. 2011; Renton *et al*. 2011). The 20%-80% of familial and 5%-15% of sporadic ALS and FTD patients show the expansion with a range of 700-1600 repeats, whereas the normal healthy individuals have less than 30 repeats (De Jesus-Hernandez *et al*. 2011; Renton *et al*. 2011; Smith *et al*. 2013). C9ORF72 is composed by 12 exons, whereof 2 alternative non-coding first exons with the hexanucleotide repeat region localized within (De Jesus-Hernandez *et al.* 2011). Therefore, depending on the alternative transcription initiation, the repeat can be on the promoter of transcript variant 1 or in the intron 1 of transcript variant 2 and 3. C9ORF72 RNA was found in different CNS tissues, through analysis of expression array data (Renton *et al*. 2011). A recent work showed that C9ORF72 protein can have a function in the regulation of

intracellular trafficking processes of the endosomal and autophagy-lysosomal compartments (Farg *et al*. 2014). Similarly to what reported for CHMP2B, in neuronal cell lines and primary cortical neurons, C9ORF72 colocalizes with four Rab proteins involved in endosomal trafficking, with a higher colocalization with Rab7 that is implicated in the biogenesis of lysosomes, the transport of endosomes and the maturation of autophagosomes (Gutierrez *et al*. 2004). So far the pathological mechanism involved in the presence of the hexanucleotides repeats is not known, however some studies found that the repeats transcripts tend to accumulate and create structure in the nucleus called RNA foci, which can produce neurodegeneration through a toxic effect (De Jesus-Hernandez *et al*. 2011). The repeat-associated non-ATG (RAN) translation can aberrantly express these transcripts with all the possible reading frame, producing poly(GP), poly(GA) and poly(GR) proteins. Inclusions in neurons composed by RAN proteins are considered an hallmark of the disease (Mori *et al.* 2013b; Ash *et al*. 2013) RNA foci could also sequester important proteins, causing alterations or dysfunctions inside the cells (Mori *et al*. 2013a; Reddy *et al*. 2013; Xu *et al*. 2013; Lee *et al.* 2013; Gendron *et al*. 2013). A recent work described a molecular mechanism for ALS/FTD with hexanucleotide repeats in C9ORF72. The mechanism consists in the alteration of RNA transcription, with transcriptional pausing and abortion due to the DNA and RNA-DNA structure formed in the repeat expansion regions. The accumulation of abortive transcripts with hexanucleotides repeats, creates G-quadruplexes and hairpins structures that lead to the binding of essential proteins, causing nuclear stress and further defects (Haeusler *et al*. 2014).

1.1.2 Frontotemporal Dementia in an RNA Perspective

An emerging branch of research in neurodegeneration is focusing on the process by which RNA could influence and lead to dysfunctions or neuronal death. For several diseases, alterations in RNA splicing, in non-coding RNA and RNA binding protein can accompany or drive neurodegeneration in different ways (Gallo *et al*. 2005; Cooper *et al.* 2009; Lagier-Tourenne *et al.* 2010). In the attached manuscript, we collected all the information related to the genes involved in frontotemporal dementia, focusing on their possible interactions

and regulations played by RNA. The review provides a broad summary of the recent literature in order to identify a common network of combined regulations, with an RNA perspective. RNA and its processing could have a key role for the unique integration of all the known genes involved in FTD and for the possible explanations of their pathological role or dysfunction mechanisms. The long non coding RNA regulations is still at the beginning phase and further work has to be done, however a global picture of interactions could help to open new questions, different experimental approaches to better decipher the signs of neurodegeneration. Please refer to the attached review "**A network of RNA and protein interactions in Fronto Temporal Dementia**" pubblished in **Frontiers in Molecular Neuroscience** in March 2015**.**

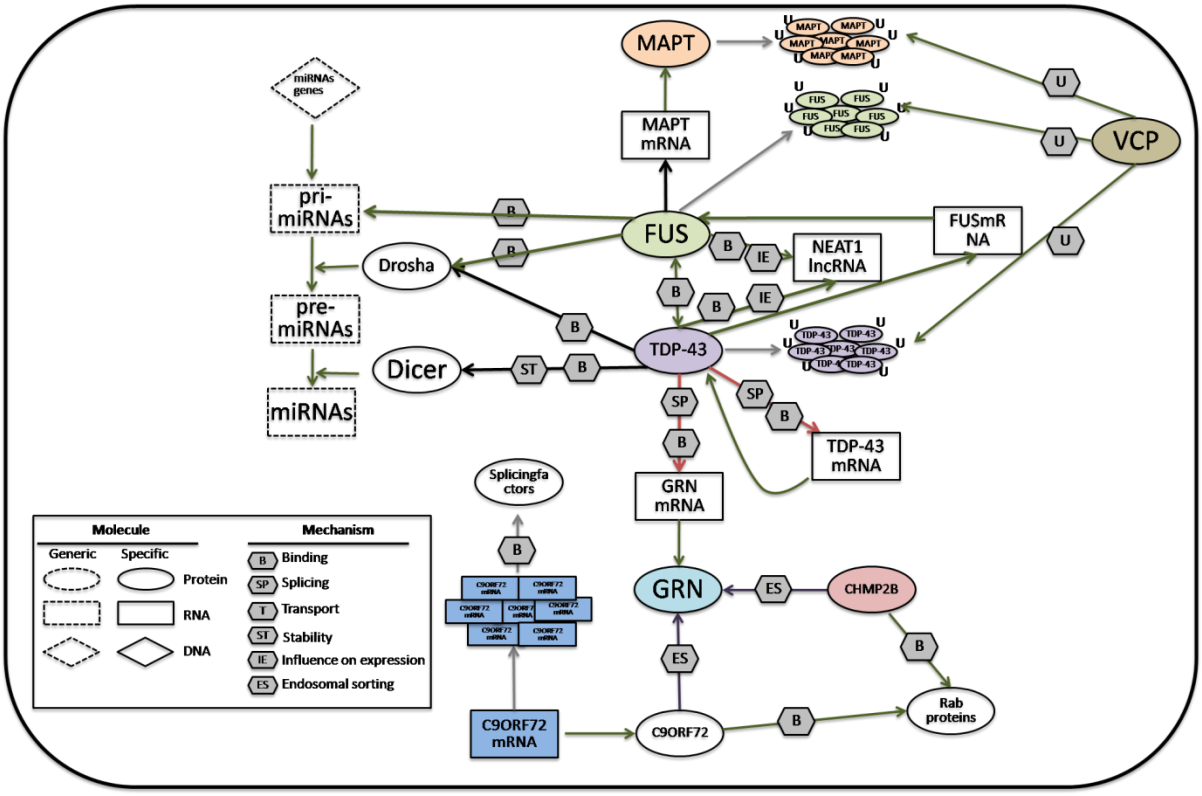


Figure 1. Network of possible interactions between proteins and RNAs, at the basis of Fronto Temporal Dementia. Green arrows indicate binding interactions or processes that result in activation or increased expression. Red arrows indicate binding interactions or processes that result in inhibition of activity or reduced expression. Black arrows indicate binding interactions or processes whose result can be either positive or negative. Purple arrows indicate binding interactions or processes which are hypotetical. Symbols as in Legend. lncRNAs= long non coding RNA. TDP-43= TAR DNA binding protein. FUS= Fused in Sarcoma. GRN=progranulin. MAPT= Microtubule-Associated Protein Tau. VCP= Valosin

Containing Protein. C9ORF72=. CHMP2B= Charged multivesicular body protein 2b. Rab7=Ras-related protein 7 (Fontana and Siva 2015).

1.2 GRN and Progranulin Structure

GRN is a gene localized on the long arm of chromosome 17, at the locus q21.31, with a distance of 1,7 Mb from MAPT (Cruts *et al*. 2006; Baker *et al*. 2006). The gene is composed by 13 exons. Exon 1, part of exon 2 and the entire 13 are non-coding (Nicholson *et al*. 2011). GRN encodes for a precursor protein of 593 amino acids and 68,5 kDa called progranulin. This protein was discovered in an independent way by different investigators that used several names such as granulin-epithelin precursor, prostate cancer, proepithelin, acrogranin and prostate cancer cell derived growth factor (He and Bateman, 2003). Progranulin can be N-glycosylated at five potential sites and secreted as a mature protein of 88 kDa (Songsrirote *et al.* 2010; Chen-Plotkin *et al.* 2010). As represented in **Figure 2** the protein is formed by 7.5 cysteine-rich granulin domains, separated through linker sequences that contain disulfide bridges (He and Bateman, 2003). The intra-linker sequences can be cleaved to produce seven non identical granulins containing cysteine rich motif and a range size from 6 to 25 KDa. The cleavage is made by different proteases such as matrix metalloproteinase-14 (Butler *et al*. 2008), elastase (Zhu *et al*. 2002), proteinase 3 and neutrophil elastase (NE) (Kessenbrock and Froehlich, 2008). The full-length progranulin, once secreted, is protected from cleavage by the high-density lipoprotein (HDL)/Apolipoprotein A-I complex (Okura *et al*. 2010) and the secretory leukocyte protease inhibitor (SLPI) (Zhu *et al.* 2002).

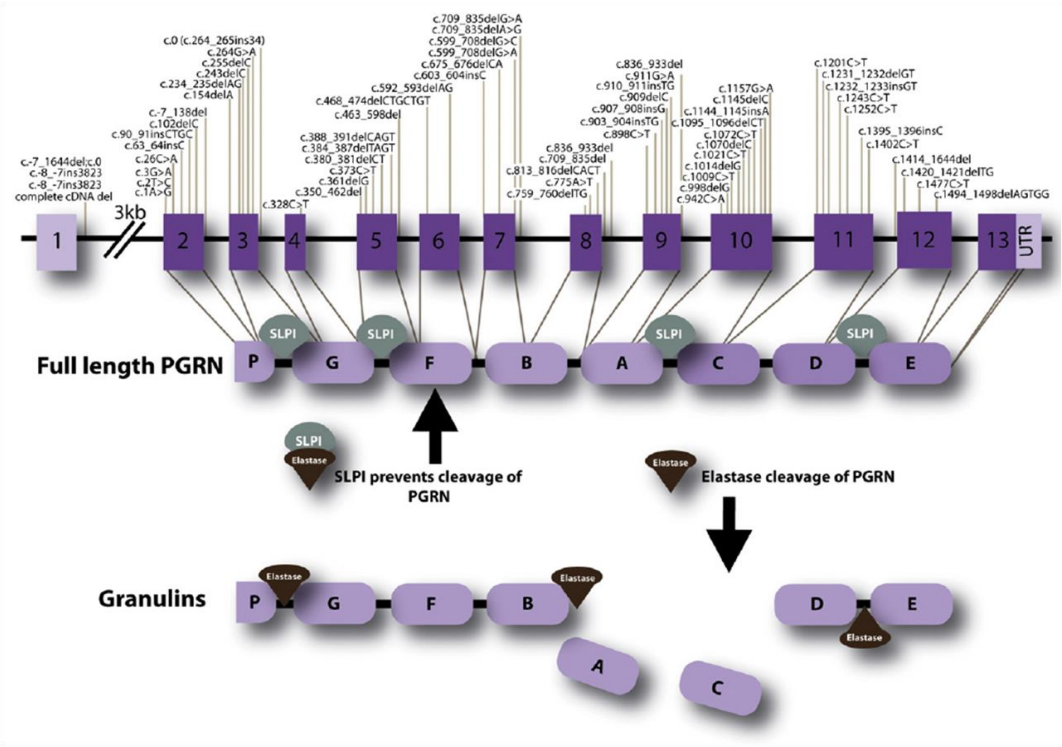


Figure 2. Schematic representation of progranulin structure. GRN gene encodes for a protein of 593 amino acids composed by intra-linked granulin peptides that can be cleaved by protease such as elastase or protect in the full-length PGRN conformation through the presence of protease inhibitor such as the secretory leukocyte protease inhibitor (SLPI) (Gass *et al*. 2012).

1.2.1 Progranulin Localization and Function

Progranulin and its fragments are both expressed in different tissues of the periphery, as well as in the central nervous system (Nicholson *et al*. 2011), especially in cerebral cortex, hippocampus and cerebellum (Daniel *et al.* 2000; Ahmed *et al*. 2007). In the brain, progranulin is expressed in different levels in neurons and microglia. In neurons the expression of progranulin increases with age, while in glial cells the level of progranulin depends on cells state of activation. Indeed an upregulation of proganulin is part of the proliferative and inflammatory response (Petkau *et al.* 2010). The subcellular location of progranulin seems to be the endoplasmic reticulum (ER) and Golgi, where it is particularly abundant in mouse cortical neurons and microglia (Almeida *et al.* 2011).

Progranulin has an N-terminal signal peptide and several N-glycosilation sites, it is usually secreted and found in serum and cerebrospinal fluid of animals, for these reasons

several groups have concentrated their attention to search for potential progranulin receptors. Sortilin (SORT1), the neuronal growth factor receptor (Jansen *et al*. 2007; Teng *et al.* 2005) has been identified as a receptor of progranulin by two different studies (Carrasquillo *et al*. 2010; Hu *et al*. 2010). Indeed a knockdown of SORT1 in HeLa cells increased the extracellular levels of progranulin, while overexpression of SORT1 led to a reduction of progranulin levels (Carrasquillo *et al.* 2010). Progranulin is able to directly interact with SORT1 on the surface of neuronal cells. Moreover it was demonstrated that SORT1 could mediate progranulin levels in mouse brain (Hu *et al*. 2010). However another potential identified progranulin receptor is the tumor necrosis factor receptor (TNFR), suggesting a role for progranulin as TNFα antagonist (Tang *et al*. 2011).

Progranulin is implicated in a wide range of biological processes such as embryogenesis (Diaz-Cueto *et al*. 2000; Daniel *et al*. 2003; Bateman and Bennet, 2009), cell survival and cell growth (Plowman *et al*. 1992; He and Bateman, 1999), inflammation and wound repair (Zhu *et al*. 2002; Kessenbrock and Froehlich, 2008; Yin *et al*. 2010), transcriptional repression (Hoque *et al*. 2003; Hoque *et al*. 2010) and several reports suggest its role in neuronal development (Van Damme *et al.* 2008). Interestingly, progranulin and the proteolytically cleaved granulins can have coherent functions, such as in the regulation of neurite outgrowth (Van Damme *et al.* 2008), or they can have contrasting roles, since granulin were found to have both agonistic and antagonistic role in cellular proliferation (Nicholson *et al*. 2011) and a pro-inflammatory function in inflammation processes (He and Bateman, 2003).

1.2.2 *GRN* Mutations Causing FTLD-TDP

In 2006 mutations found on GRN were discovered to be the cause of FTLD, with ubiquitinated positive inclusions (Baker *et al*. 2006). So far 69 different GRN mutations have been discovered in 231 families (Cruts *et al*. 2012). The GRN mutations frequency ranges from 1 to 11,7% in FTD patients, but the frequency rises to 12-25% in familial FTD (Cruts *et al*. 2006; Gass *et al*. 2006; Huey *et al*. 2006; Bronner *et al*. 2007; Borroni *et al*. 2008). The majority of GRN mutations are nonsense, frameshift and splice site that cause a premature stop codon (Cruts *et al*. 2006; Baker *et al*. 2006). However missense mutations with a partial

decrease of progranulin and a loss of its function (Mukherjee and Pastor, 2006; Mukherjee *et al*. 2008; Shankaran *et al*. 2008; Wang *et al*. 2010), silent and intronic mutations with unknown pathology can also occur. Usually all these type of mutations lead to a decrease of GRN through nonsense-mediated mRNA decay and subsequent haploinsufficiency (Baker *et al*. 2006; Cruts *et al*. 2006). However GRN mutations can cause a reduction of cellular progranulin levels through additional mechanisms, such as a decrease observed in the amount of secreted protein due to the presence of mutations that affect the signal peptide sequences (Gass *et al.* 2006; Le Ber *et al*. 2008) or a loss of GRN mRNA translation due to mutations located in the Kozak sequence of the GRN gene (Baker *et al*. 2006; Cruts *et al*. 2006; Gass *et al*. 2006; Le Ber *et al*. 2008). In any case the result is a reduction of progranulin levels that can also be observed in either serum or cerebrospinal fluid (CSF) of GRN mutation patients, where progranulin is ~30-50% of the normal amount (Van Damme *et al*. 2008). A decrease in progranulin levels was also measured in plasma of GRN mutation patients (Finch *et al*. 2009) and a reduced GRN mRNA level was detected in patient whole blood samples (Coppola *et al*. 2008).

The major component of the ubiquitinated positive inclusions found in GRN mutation patients with FTLD-U is TDP-43, subsequently termed FTLD-TDP (Mackenzie *et al*. 2006, Mackenzie *et al*. 2010; Sampathu *et al*. 2006). The relation between TDP-43 and progranulin is not fully understood, however recent works indicated that TDP-43 has a post-transcriptional regulation on GRN mRNA. TDP-43 was shown to bind GRN 3'UTR, controlling the stability of GRN mRNA and the level of progranulin protein (Polymenidou *et al*. 2011; Colombrita *et al*. 2012). Moreover a knock-down of TDP-43 in mice showed an increase in the amount of GRN mRNA level (Polymenidou *et al*. 2011; Colombrita *et al*. 2012). The relation between GRN and TDP-43 was also demonstrated in vitro: cells that were treated with siRNA against GRN for 72h, showed a caspase-dependent cleavage of TDP-43 into fragments (Zhang *et al*. 2007); whereas primary neuronal cultures upon knowckdown of GRN showed a re-localization of TDP-43 in the cytoplasm (Guo A. *et al*. 2010).

1.3 *MAPT* and Tau Structure

The microtubule associated protein tau, termed MAPT gene is located on the chromosome 17 at the locus q21.3. The gene contains 16 exons, out of which 11 are expressed in CNS (Wolfe *et al*. 2012). The primary transcript contains 13 exons with eight constitutive exons: 1, 4, 5, 7, 9, 11, 12, 13, three alternative spliced exons: 2, 3, and 10 and two transcribed but not translated exons: -1 and 14 (Luna-Munoz *et al.* 2013). The extensive alternative splicing process gives rise to six different mRNAs, translated in six different isoforms present in the CNS. *MAPT* gene encodes for tau protein, whose largest isoform is composed by of 441 amino acids. The isoforms differ for 29 amino acids encoded by exon 2 or exon 3 and the presence of three (3R) or four (4R) C-terminal repeat-regions, according to the presence or the absence of exon 10 that encodes for the additional microtubule binding domain. Indeed as reported in **Figure 3**, the tau protein structure contains in the C-terminal part several repeated microtubule binding domains of 18 residues that regulate the rate of microtubules polymerization. Whereas the N-terminal projection domain includes an acid and proline rich regions which interacts with cytoskeletal elements (Luna-Munoz *et al*. 2013).

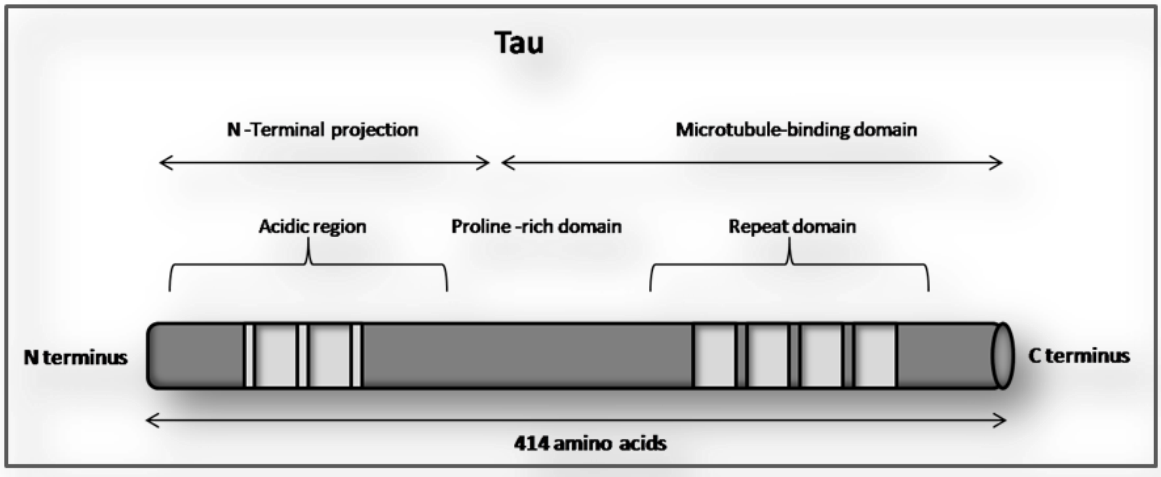


Figure 3. Schematic representation of tau functional domains (the largest isoform, 441 amino acids).The projection domain, including an acidic and a proline-rich region, interacts with cytoskeletal elements. The N-terminal part is also involved in signal transduction pathways by interacting with proteins such as PLC-γ and Src-kinases. (manuscript Fontana, Siva, Denti 2015).

1.3.1 Tau Localization and Function

Tau is expressed in different tissues and highly enriched in neurons in the CNS. Indeed considering its association and interaction with microtubules that are particularly important for neuronal structure and function, tau is primarily found in axons and also in dendrites (Binder *et al*. 1985; Ittner *et al*. 2010). All the six isoforms are expressed in adults and the shorter tau isoform, containing three microtubule binding domains and the skipping of exons 2 and 3 is expressed also in fetal brain (Goedert *et al*. 1989; Goedert and Jakes 1990).

It has been reported that tau can be secreted unconventionally in naked form (Chai *et al.* 2012) or associated to exosomes (Saman *et al*. 2012) and/or to other membrane vesicles (Simón *et al*. 2012). Detectable level of tau was also measured in the CSF of human tau transgenic mice (Andorfer *et al*. 2003; Yamada *et al*. 2011) and comparable levels was found in the CSF of healthy individuals (Hampel *et al*. 2010), leading to the hypothesis that tau release occurs under physiological conditions in vitro and in vivo. It is still unclear if the secretion of tau could have a physiological or pathological role. However the unconventional secretion of tau species could represent the way required for spreading and transmission of tauopathy observed in mice brain (Clavaguera *et al*. 2009; Chai *et al*. 2012).

Tau has a role in the assembly, stabilization and dynamic of the microtubular network, particularly important for the neuronal structural integrity of the cytoskeleton (Fellous *et al*. 1977). Indeed microtubules are important for the neuronal function, creating the structure for the axonal process growing from the neuronal cell body and forming a path for the transport of material to the synapses (Wolfe *et al*. 2012). An increased level of tau expression was indeed observed at the synapses, suggesting a role in the axonal transport (Mandell and Banker, 1996). Tau was also shown to regulate the axonal transport of organelles, including mitochondria (Medina and Avila, 2014). Tau protein can be phosphorylated at many different sites, both in physiological and pathological conditions (Johnson and Stoothoff, 2004). The alternative phosphorylation can control its biological function to interact with microtubules or its ability to self-assemble into neuronal filaments during neurodegenerative disorders. Different kinases show the capability to phosphorylate

tau in vitro, however the process in vivo seems quite unclear (Mandelkow *et al*. 2004; Doble and Woodgett, 2003). Phosphatases such as PP1 and PP2A can dephosphorylate tau and the balance between kinase or phosphatase activity will determine tau functionality.

1.3.2 *MAPT* Mutations in FTLD

In frontotemporal dementia (FTLD-Tau), patients show inclusions in neurons and glia of hyperphosphorylated tau called neurofibrillary tangles (NFT) (Lee *et al*. 2005). However if the hyperphosphorylation of tau precede or is a consequence of the filament assembly and which site is relevant for the pathology is still not clear (Wolfe *et al*. 2012). It was reported that through its tandem repeats, with the amino-terminal and the carboxy-terminal part, tau can assemble into filaments (Wischik *et al*. 1988; Wegmann *et al*. 2013). These filaments can also be assembled from non-phosphorylated full length recombinant tau with the interaction of negatively charged compounds such as free fatty acids and RNA (Kampers *et al*. 1996; Wilson *et al*. 1997). However the tangles found in patient neurons consist of hyperphosphorylated aggregates of insoluble tau. A direct toxic effect of these inclusions and/or a loss of the axonal functionalities of tau have been considered to contribute to the disease. So far 44 different MAPT mutations have been discovered in FTD population (Cruts *et al.* 2012). These are all missense mutations, splice mutations or both and the major part is located in the region encoding the C-terminal part or nearby exon 10. Therefore most of the mutations are affecting the correct ability to bind and promote the microtubule assembly (Goedert and Jakes, 2005). Approximately half of the mutations associated with exon 10 are altering the splicing of this exon, causing an increase of the ratio 4R to 3R. In neurons this ratio is always controlled and an imbalance of 4R to 3R can lead to neurodegeneration and the assemble of 4R isoforms into filaments (Wolfe *et al*. 2012; Spillantini and Goedert, 2013). Mutations located close to exon 10 5’ splice site could increase the inclusion of exon 10, either by altering the linear cis-splicing elements or through a destabilization of a stem-loop structure localized at the exon-intron junction (Spillantini and Goedert, 2013; Grover *et al*. 1999; D’Souza *et al*. 1999). The stem-loop is originated from the self-complementarity between bases located in that region and it has a role in masking the 5’ spice site. Therefore mutations that disrupt the stem-loop make the 5’

splice site more accessible to splicing factors that lead to the inclusion of exon 10 (Wolfe *et al.* 2012). Interestingly, silencing of FUS (another protein involved in the pathology of FTD, previously described) was reported to alter the splicing of MAPT. In particular, FUS was shown to help the skipping of MAPT exon 10 in primary cortical neurons (Ishigaki *et al*. 2012).

1.4 miRNAs Biogenesis and Function

microRNAs (miRNAs) are a group of small non-coding RNAs with important regulatory roles on the post-transcriptional expression of target mRNAs (Bartel, 2009; Ghildiyal and Zamore, 2009), they are identified in animals, plants, green algae and viruses (Griffiths-Jones *et al*. 2008). To date more than 10000 miRNAs have been found, around 940 of which are in the human organism (Sayed *et al.* 2011). Specifically miRNAs are 18-22 nt-long single-stranded RNA molecules, firstly discovered by Lee *et al*. (1993), generated from longer transcripts of different lengths (pri-miRNA), usually transcribed by RNA polymerase II, from intragenic or intergenic DNA regions (Lee *et al*. 2004; Garzon *et al*. 2010). However some miRNAs could also be transcribed by RNA polymerase III as reported by Borchert and collegues (Borchert *et al*. 2006). The primary transcript termed as primary miRNA (pri-miRNA) transcript is processed by the micro-processor complex in the nucleus. The microprocessor complex is composed by an RNase III enzyme, Drosha and its cofactor Pasha found in flies or its ortholog DiGeorge syndrome critical region gene 8 (DGCR8) found in mammals and Caenorhabditis elegans (Han *et al*. 2006). The process lead to the cleavage of pri-miRNA and formation of a small hairpin structure of 70-100 nt called precursor miRNA (pre-miRNA). Afterwards the pre-miRNA is exported to the cytoplasm through a RanGTP-dependent double stranded RNA binding protein called Exportin 5. In the cytosol the pre-miRNA is further processed by Dicer (Kim *et al*. 2005), an RNAse-III nuclease and additional proteins such as the transactivation response RNA-binding protein (TRBP) and the protein activator of the interferon-induced protein kinase PKR (PACT). Dicer produces an imperfect double-stranded RNA (Hutvagner *et al*. 2001, Lee *et al.* 2004). Subsequently, the RNA duplex is unwound by an RNA helicase and one strand is loaded on the RNA-Induced Silencing Complex (RISC) and associates with Argonaute-2 (Ago2; Meister *et al*. 2004 a and b). The other strand was believed to be degraded (Chendrimada *et al*. 2005), however recent studies demonstrated that can associate with different Ago protein and have a role in downregulation of target mRNA (Czech and Hannon, 2011). In any case the procedure of strand selection is not yet fully clarified. The miRNA-RISC complex interacts with the target mRNAs using classic base-paring. The binding involved the seed region of the miRNA, localized on its 5' end between nucleotides 2 to 8 (Bartel, 2009). However a significant fraction of non-canonical interactions, that involve non-seed base pairing can occur

between the miRNA-RISC complex and the target mRNA (Helwak *et al*. 2013). Frequently these sequences are located in the 3'untranslated region (UTR) of target mRNAs, however they were subsequently localized in the coding region and the 5'UTR of a mRNA (Rigoutsos, 2009; Orom *et al*. 2008). The RISC complex induces mRNA downregulation through two different functional pathways: either the mRNA cleavage if there is a perfect complementarity between the sequences of the miRNA and its target mRNA, or translation inhibition in case of imperfect binding (Bartel, 2009). In animals translational repression is the most frequent way of action for miRNAs, however in case of perfect complementarity, Ago2 is the protein involved in the cleavage of the target mRNA in humans. (Liu *et al*. 2004). The whole process is summarized in **Figure 4**. The mechanisms for translational repression played by miRNAs are still unclear, however different works suggested the promotion of target mRNA deadenylation, sequestration of miRNAs and target mRNAs by stress granules and P bodies, disruption of translation initiation or degradation caused by RISC after translation (Tang *et al*. 2008). For this reasons the translational repression induced by miRNAs seems to be a complex process that lead to a fine tuning downregulation effect at the protein level. miRNAs modulate through their binding the transcriptome of cells (Guo H. *et al*. 2010). Indeed one single miRNA could potentially target hundreds of different mRNAs and one single mRNA could be controlled by many different miRNAs. Moreover miRNAs themselves can be regulated post-transcriptionally as suggested by Farajollahi and Maas (Farajollahi and Maas, 2010), creating an increasingly intricate network of regulation.

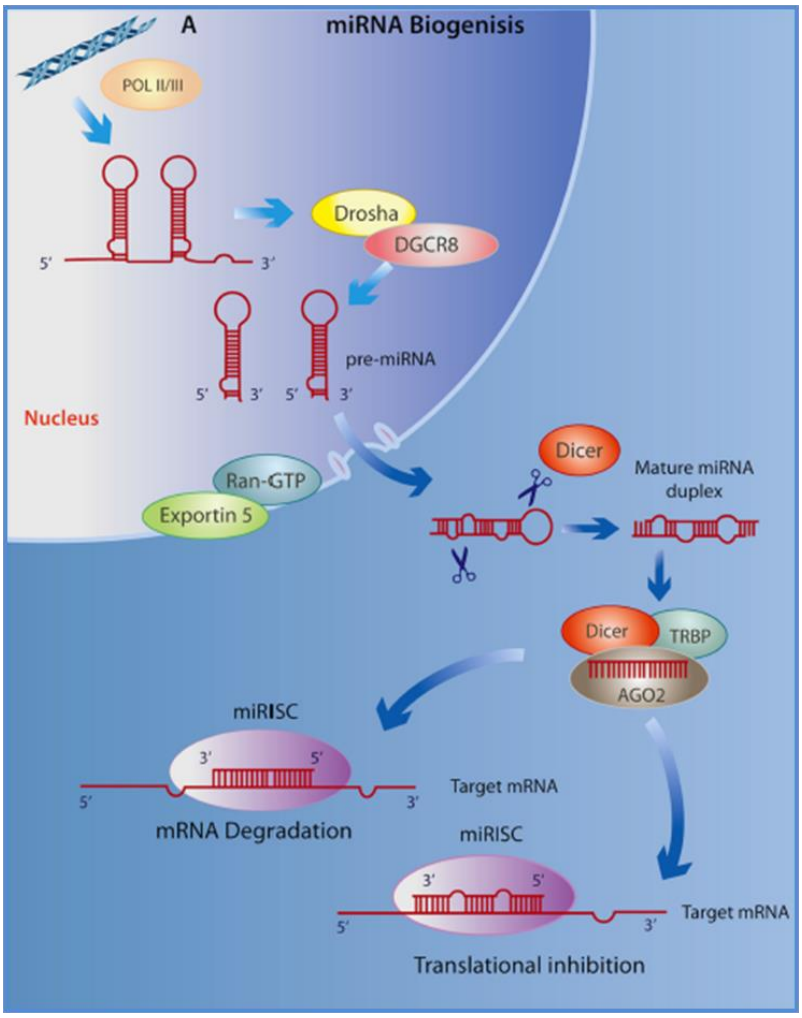


Figure 4. Scheme of miRNAs biogenesis. The primary transcript (pri-miRNA) is cleaved by Drosha and DGCR8, generating a miRNA precursor (pre-miRNA) that is exported to the cytoplasm through Exportin5 and Ran-GTP61. Dicer and TRBP process the pre-miRNA to produce the mature miRNA, that is incorporated to the RISC and subsequently binds to the 3'UTR of a target mRNA leading to either mRNA degradation or translational repression. (Adapted from Sapadaro and Bredy, 2012)

1.4.1 Non-Canonical Biogenesis

Latest findings revealed that many miRNAs are generated through an alternative process that does not require the action of Drosha. These miRNAs (dubbed "mirtrons") are encoded by region located on intron or intron-exon junctions. In general they are short introns with hairpin potential that can be spliced and exported out of the nucleus. After the nuclear export the mirtrons follow the canonical miRNA pathway, with the cleavage of Dicer and

incorporation into the silencing complex (Okamura *et al*. 2007; Berezikov *et al*. 2007). Originally mirtrons were identified in flies and worms, however similar loci were recognized in rodents and primates (Berezikov *et al*. 2007; Babiarz *et al*. 2008). These loci give rise to shorter precursor stem lengths, lacking the lower stem which usually recruits Drosha/DGCR8 for the cleavage (Han *et al*. 2006). As depicted in **Figure 5**, in all the spliced intron, the initial product is a lariat that is processed by a debranching enzyme, and subsequently transported as pre-miRNA to the cytoplasm through Exportin-5. However some mirtron loci are not located at a distance from splice site and the small RNA-generating hairpin is at one end of the intron. These "tailed" mirtrons have an unstructured extensions at the 5' or 3' to the hairpin (Ruby *et al.* 2007; Babiarz *et al.* 2008). The 3'tailed mirtron have a tail extension to the splice acceptor site, such as reported for miR-1017 sequence (Flynt *et al*. 2010). During the biogenesis the 3' tail is trimmed in the nucleus by RNA exosome, after the splicing and debranching processes, as reported in the **Figure 5**. Whereas the biogenesis of 5' talied mirtrons has not been investigated in details, even though a number of 5' tailed mirtrons have been identified in various mammals (Barbiaz *et al.* 2008; Chiang *et al*. 2010).

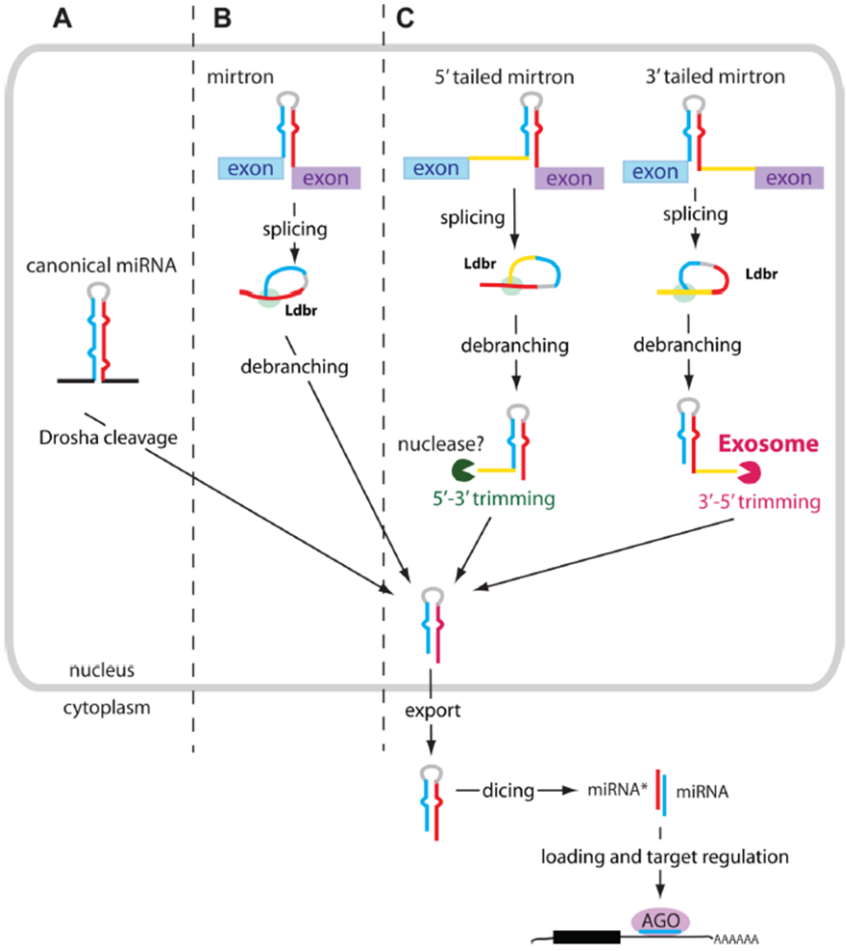


Figure 5. Representation of the canonical miRNAs and mirtrons biogenesis pathways. A) Canonical miRNAs biogenesis, through Drosha cleavage of pri-miRNA transcripts. B) Lariat debranching enzyme (Ldbr) debranch the spliced introns to produce pre-miRNA hairpins. C) Tailed mirtrons are spliced, debranched and the tails is trimmed by an RNA exosome in case of 3'tails. 5' trimming is performed by unknown enzymes. The pre-miRNA hairpins generated from these alternative pathways are export to the cytoplasm where they undergo the usual process for the production of a mature and active miRNAs (Westholm and Lai, 2011).

1.4.2 miRNAs in Neurodegenerative Diseases

The assumption that miRNAs dysregulation could lead to neurodegeneration derived from different experiments in which knockout of Dicer, with consequent disruption of miRNAs biogenesis causes neurodegenerative phenotypes. In particular, deletion of Dicer was performed in mouse cerebellar neurons (Schaefer *et al*. 2007), in midbrain dopamine neurons (Kim *et al.* 2007), in striatal, retinal, spinal and cortical neurons (Cuellar *et al.* 2008;

Damiani *et al*. 2008; Davis *et al.* 2008; Haramati *et al*. 2010) and in glial cells (Tao *et al*. 2011; Shin *et al*. 2009; Wu *et al*. 2012). Several studies showed that the DGCR8 haploinsufficiency leads to a decreased production of miRNAs, with neuronal alteration as result (Stark *et al.* 2008; Fenelon *et al*. 2011; Schofield *et al*. 2011). Hebert and colleagues found an altered phosphorylation pattern of tau after the deletion of Dicer, suggesting the presence of miRNAs' control on specific aspects of the neurodegeneration process (Hebert *et al*. 2010).

Microarray analyses demonstrated a specific brain expression of different miRNAs (Lim *et al*. 2005; Manakov *et al*. 2009), which is particularly relevant during mouse or zebrafish brain development (Miska *et al*. 2004; Kapsimali *et al*. 2007), such as miR-17-5p, miR-199a, miR-92 found important and higher expressed during the embryonic and post-natal days, while miR-29b, miR-138 were more expressed in the adult stages of mouse development (Miska *et al.* 2004). Sequencing data lead to the development of a mammalian miRNAs expression atlas in different cell types (Landgraf *et al*. 2007). In particular the neuronal specific miRNAs clusters miR-9 and miR-124 or the miRNAs clusters miR-15a and miR-126 were found abundant in the adult frontal cortex, cerebellum, hippocampus regions and higher expressed in neuroblastoma cell lines such as SH-SY5Y (Landgraf *et al*. 2007). Recent studies showed an interesting correlation between the expression pattern of miRNAs in brains of different primates and human development (Somel *et al*. 2011 and 2010; Hu HY *et al.* 2011) suggesting that changes in miRNAs profiles induced significant differences at mRNA and protein levels between human and other primates' brain. Therefore, not only the global loss of miRNAs can cause neurodegeneration, but in some cases a specific alteration of a single miRNA pattern in the brain could be linked to a particular disease. For this reasons in the last years different research groups focused their efforts on exploring circulating miRNAs as possible biomarkers for neurodegenarative disorders.

For a better analysis of miRNAs dysfunctional mechanisms related to different neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis and their use as potential biomarkers, please refer to the attached chapter: "**Circulating cell-free microRNAs as biomarker for neurodegenerative diseases**", accepted for publication in the book "**Mapping Nervous System Diseases via MicroRNAs**". The volume will appear in a new book series "**Frontiers in Neurotherapeutics**" by CRC Press-Taylor & Francis Group (Boca Raton FL, USA).

CHAPTER TWO: MATERIALS AND METHODS

2.1 Cloning of Reporter Vectors

GRN 3'UTR was amplified through RT-PCR, starting from RNA extracted from HeLa cells and retro-transcribed into cDNA. The full length GRN 3'UTR of 304 bp was amplified using GRN Forward and GRN Reverse Primers containing restriction sites for XbaI enzyme (refer to **table 2.1a**). The amplified product of 304 bp was purified using PCR purification kit from Qiagen and cloned inside pmirGLO plasmid vector, in the multiple cloning site, located at the 3' of firefly luciferase (**Figure 2.1**). The cloning was performed through the sticky ends produced with XbaI digestion. pmirGLO plasmid (from Promega) is a dual-luciferase plasmid containing both firefly luciferase gene (*luc2*), under the control of PGK promoter, and Renilla luciferase gene, under the control of SV40 promoter. The vector is designed to measure miRNAs activity, through the inclusion of the putative miRNA binding sites or the entire 3'UTR of interest at the 3' of *luc2.* Firefly luciferase assay is used to analyze mRNA regulation, while *Renilla* luciferase is a control reporter for normalization.

In order to investigate the effect of miRNAs on different parts of GRN 3'UTR, the GRN 3'UTR full lenght was firstly divided in two parts, using the restriction site for SacI, located after 114 bp. The first part of GRN 3'UTR of 114 bp was called GRNIPart, while the second part of 190 bp was called GRNIIPart. Following the digestion with SacI, the products were run on agarose gel and extracted through gel extraction kit from Qiagen. The samples were then purified using PCR purification kit from Qiagen and used for the cloning procedure. GRNIPart and GRNIIPart were both cloned inside pmirGLO plasmid vector at the multiple cloning site, using sticky ends derived from the digestion with XbaI and SacI respectively.

The fragment GRNIPart was further divided in two different parts, using specific primers for the amplification of the desired products. GRN Forward and RevIGRNa Primers were used for the amplification of the first 65 bp product called GRNa fragment. ForIIGRNb and RevIIGRNb Primers were used for the amplification of the 61 bp product called GRNb fragment (refer to the **table 2.1a** for primer sequences). GRNa and GRNb products were amplified, purified through PCR purification kit (Qiagen) and cloned inside pmirGLO vector, using XbaI and SalI restriction sites.

MAPT 3'UTR was amplified from human genomic DNA using MAPT Forward and MAPT Reverse Primers, containing SacI and NheI restriction sites (refer to the **table 2.1b** for primer sequences). The amplified product of 4167 bp was purified using PCR purification kit from Qiagen and cloned inside pmirGLO plasmid vector, in the multiple cloning site, located at the 3' of *luc2*.

MAPT 3'UTR was further divided into 8 different partially overlapped fragments of 700 bp. Amplification of the fragments was obtained with specific primers reported in **table 2.1b.** The different fragments were cloned inside pmirGLO vector, using SacI and NheI or XbaI and SalI restriction sites.

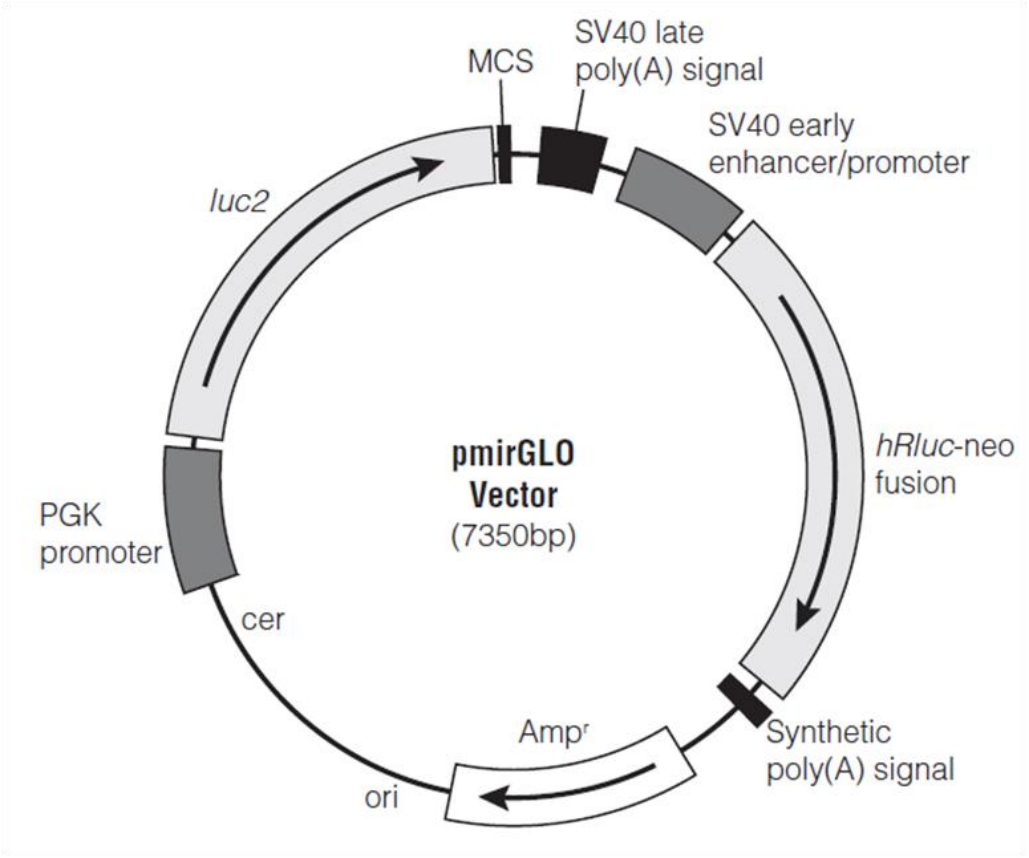


Figure 2.1. Map of pmirGLO Vector with specific features (Promega)

Table 2.1a

GRN PRIMERS	SEQUENCES
GRN Forward Primer	5’- AAATCTAGAGGGACAGTACTGAAG -3’
GRN Reverse Primer	5’- ATCTAGAGAAAGTGTACAAACTTTATTG-3’
RevIGRNa	5’- AAAGTCGACAGGCCTGAGCAGA- 3’
ForIIGRNb	5’-AAATCTAGACCTCCCTAGCACCTCC-3’
RevIIGRNb	5’- AAAGTCGACGTGATGGGGAGCTCA- 3’

Table 2.1b

MAPT PRIMERS	SEQUENCES
MAPT Forward primer	5'-AAAGAGCTCTTTGTGATCAGGCCC-3'
MAPT Reverse primer	5’-AAAGCTAGCAATCAGAGTAATAACTTTATTTCC-3’
MAPT Reverse I	5'-ATAGCTAGCACTGACCCACAGCAG-3'
MAPT Forward II	5'-ATAGAGCTCTGAGTGTGACGGGG-3'
MAPT Reverse II	5'-ATAGCTAGCTGGGAATTCGGGACA-3'
MAPT Forward III	5'-ATAGAGCTCCTAACCAGTTCTCTT-3'
MAPT Reverse III	5'-ATAGCTAGCCTGATACTATGCATG-3'
MAPT Forward IV	5'-ATAGAGCTCGAGAGAGCCCTTTCC-3'
MAPT Reverse IV	5'-ATAGCTAGCACCTAGTCTGTGCCC-3'
MAPT Forward V	5'-ATAGAGCTCACGAGGTGTCTCTCA-3'
MAPT Reverse V	5'-ATAGCTAGCCAAACCAGAAGTGGC-3'
MAPT Forward VI	5'-GGATCTGCTC TAG AGGCCCAAG-3'
MAPT Reverse VI	5'-ATAGTCGACGGGATTGTCCTCATTT-3'
MAPT Forward VII	5'-ATAGAGCTCGGTTCCTCTTCCTGA-3'
MAPT Reverse VII	5'-ATAGCTAGCGCTTAGAGGGAAGGA-3'
MAPT Forward VIII	5'-ATAGAGCTCGGTTTCTCTTTTCCAC-3'

2.2 Cloning of Over-Expressing Plasmids

The miRNA constitutive-expression cassette has been generated by cloning a fragment containing the precursor region of miRNAs (pre-miRNA) in the psiUx plasmid (**Figure 2.2**), using BglII and XhoI or BglII and KpnI restriction sites (Denti et al., 2004). All the primers used for the amplification of fragments containing the pre-miRNA region are reported in **table 2.2**. Pre-miR-939 was amplified from human genomic DNA using 5'- ACT GCT CGA GGG TCC TCC AGA ACG TGT TCA - 3' (forward primer) and 5'- ACT GAG ATC TCG CTA CCA GGA GGA AAG CAA - 3' (reverse primer); pre-miR-659 was amplified from human genomic DNA using 5'- ACT GCT CGA GCA CTG TCA TTA TTT TCT CAC - 3' (forward primer) and 5'- ACT GAG ATC TGC GTT CTT GTT TTG TGT TTC - 3' (reverse primer); pre-miR-615 was amplified from human genomic DNA using 5'- ACT GAG ATC TCG ACG ACA TAA TTG GAT CAT - 3' (forward primer) and 5'- ACT GGG TAC CAG GAA GGG GTG AAT AGC TTG - 3' (reverse primer); pre-miR-608 was amplified from human genomic DNA using 5'- CTG AGA TCT GGT AAT GGC TCC ATC TGG AG - 3' (forward primer) and 5'- CTG CTC GAG TTG CAG ACT CTT GGG CCC TT - 3' (reverse primer). miR-181a over-expressing plasmid was already present in the laboratory. Whereas I cloned two additional over-expressing plasmids for the over-expression of miR-29a and miR-1207-5p, used for a project in collaboration with Assistant Professor Nikolaos Balatsos from the University of Athens in Greece, and an additional over-expressing plasmid for miR-612. However, these over-expressing plasmids were not used for the functional analysis of GRN and MAPT post-transcriptional regulation.

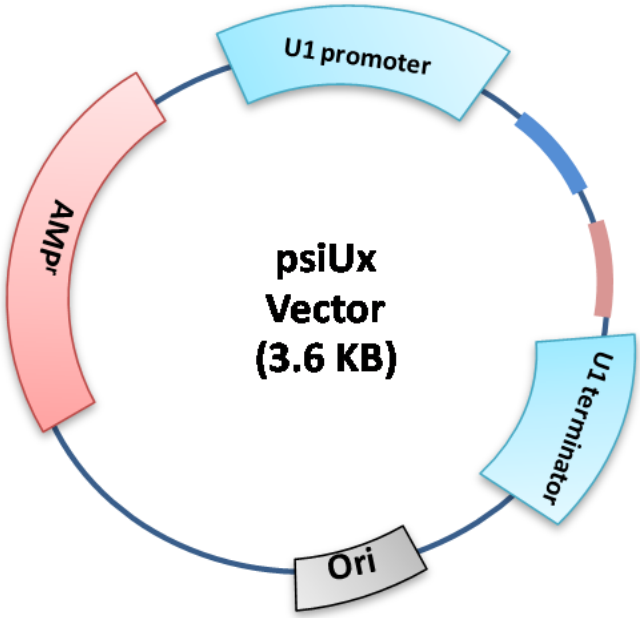


Figure 2.2 Map of psiUX vector used for the production of miRNAs over-expressing plasmid. This vector was produced and published by Prof. Michela Denti in 2004 (Denti *et al.* 2004).

Table 2.2

miRNAs PRIMERS	SEQUENCES
Pre-miR-939 Forward	5'-ACTGCTCGAGGGTCCTCCAGAACGTGTTCA-3'
Pre-miR-939 Reverse	5'-ACTGAGATCTCGCTACCAGGAGGAAAGCAA-3'
Pre-miR-659 Forward	5'-ACTGCTCGAGCACTGTCATTATTTTCTCAC-3'
Pre-miR-659 Reverse	5'-ACTGAGATCTGCGTTCTTGTTTTGTGTTTC-3'
Pre-miR-615 Forward	5'-ACTGAGATCTCGACGACATAATTGGATCAT-3'
Pre-miR-615 Reverse	5'-ACTGGGTACCAGGAAGGGGTGAATAGCTTG-3'
Pre-miR-608 Forward	5'-ACTGAGATCTGGTAATGGCTCCATCTGGAG-3'
Pre-miR-608 Reverse	5'-ACTGCTCGAGTTGCAGACTCTTGGGCCCTT-3'

2.3 Cell Cultures and Transfection Experiments

Human cervical carcinoma HeLa cell line was grown in DMEM medium (Gibco®, Life Technologies) supplemented with 2 mM L-Glutamine, Penicillin/Streptomycin and 10% Fetal Bovine Serum (FBS). Human neuroblastoma KELLY cell line was cultivated in RPMI-1640 medium (Gibco®, Life Technologies) supplemented with 2 mM L-Glutamine, Penicillin/Streptomycin and 10% Fetal Bovine Serum (FBS). Human neuroblastoma SH-SY5Y and SK-N-BE cell lines were grown in 1:1 mixture of Eagle's Minimum Essential Medium and

F12 Medium (Gibco®, Life Technologies) supplemented with 2 mM L-Glutamine, Penicillin/Streptomycin and 10% Fetal Bovine Serum (FBS). All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Cells were seeded on 24-well plate (Luciferase Assay) and 6-well plate (Western Blotting and ELISA assay). Cells were transfected at 80% confluence with Lipofectamine LTX and Plus reagent (Life Technologies) for Luciferase and ELISA Assays and with with Lipofectamine 3000 (Life Technologies) for Western Blot analysis. Whereas cells were plated on culture dishes and transfected with Lipofectamine 3000 (Life Technologies) for polysomes analysis.

2.4 miR-CATCH Experiments

2.4.1 Formaldehyde Cross-Linking and Cell Lysis

A detailed description of miR-CATCH protocol is reported in a recent paper (Vencken et al. 2014). Cultures of three selected neuroblastoma cell lines (KELLY, SK-N-BE, SH-SY5Y) were grown in T75 culture flasks. Three T75 full confluent flasks were used as a starting material for each miR-CATCH experiment. Cells (approximately $3x10^6$) were fixed with 2% formaldehyde to a final concentration of 1% for ten minutes. Formaldehyde allows all the factors, such as miRNAs and proteins to GRN and MAPT mRNAs, to be cross-linked. The reaction was stopped with the addition of 3M glycine to the final concentration of 0,2 M. Then, cells were washed several times with ice-cold DPBS and repeatedly centrifuged at 4°C for 5 minutes at 2500 g. Cell lysis was prformed with 1,4 ml of FA lysis buffer (50mM HEPES, 140 mM NaCl, 1mM EDTA, 1% v/v Triton-X100, 0,1% w/v sodium deoxycholate) with PMSF, protease inhibitors and RNase inhibitor added just before the lysis reaction. Cells were homogenized in 0,4 ml of FA lysis buffer and 0,4 ml of glass beads using the FastPrep 24 homogenizer used 4 times for 30 seconds at speed 5,5. The lysate was filtered into a second tube and glass beads were washed by adding 1 ml of FA lysis buffer, centrifuging for 5 minutes at 3500 g, 4°C. Finally cell debris was pelleted by centrifuging at 4°C for 5 minutes at 18000 g and the supernatant was transferred to a new tube.

2.4.2 Streptavidin Bead Preparation and miRNA:GRN or MAPT mRNAs Pulldown

Dynabeads MyOne Streptavidin C1 magnetic beads (Life Technologies, Grand Island, USA) were prepared to the manufacturer's instructions. An excess of 800 pmol of biotinylated capture oligonucleotides was immobilised to the magnetic beads in 1X B&W buffer (1X TE buffer, 1M NaCl), rotating the tubes for 15 minutes at room temperature. Oligo-prepared beads were subsequently washed with hybridization buffer (2X TE buffer, 1 M LiCl) and resuspended with 0,7 ml hybridization buffer, 0,5 ml 1X TE buffer and mixed with 0,2 ml of formaldehyde-treated cell lysates for 90 minutes under continuous tumbling at 37°C for adequate annealing. The beads were washed four times using washing buffers (0,1 M NaCl with 0,5% SDS and 0,1 M NaCl) and GRN or MAPT mRNA:miRNA complexes were captured using a Dynal magnet. The beads were suspended in TE buffer before heat treatment at 60°C for 5 minutes to reverse the interaction between the biotin-labelled DNA:mRNA:miRNA complexes and the magnetic beads. Cross-linked nucleic acids and proteins were incubated for 45 minutes at 70 °C to reverse the cross-linkages. Samples were subsequently used for validations studies.

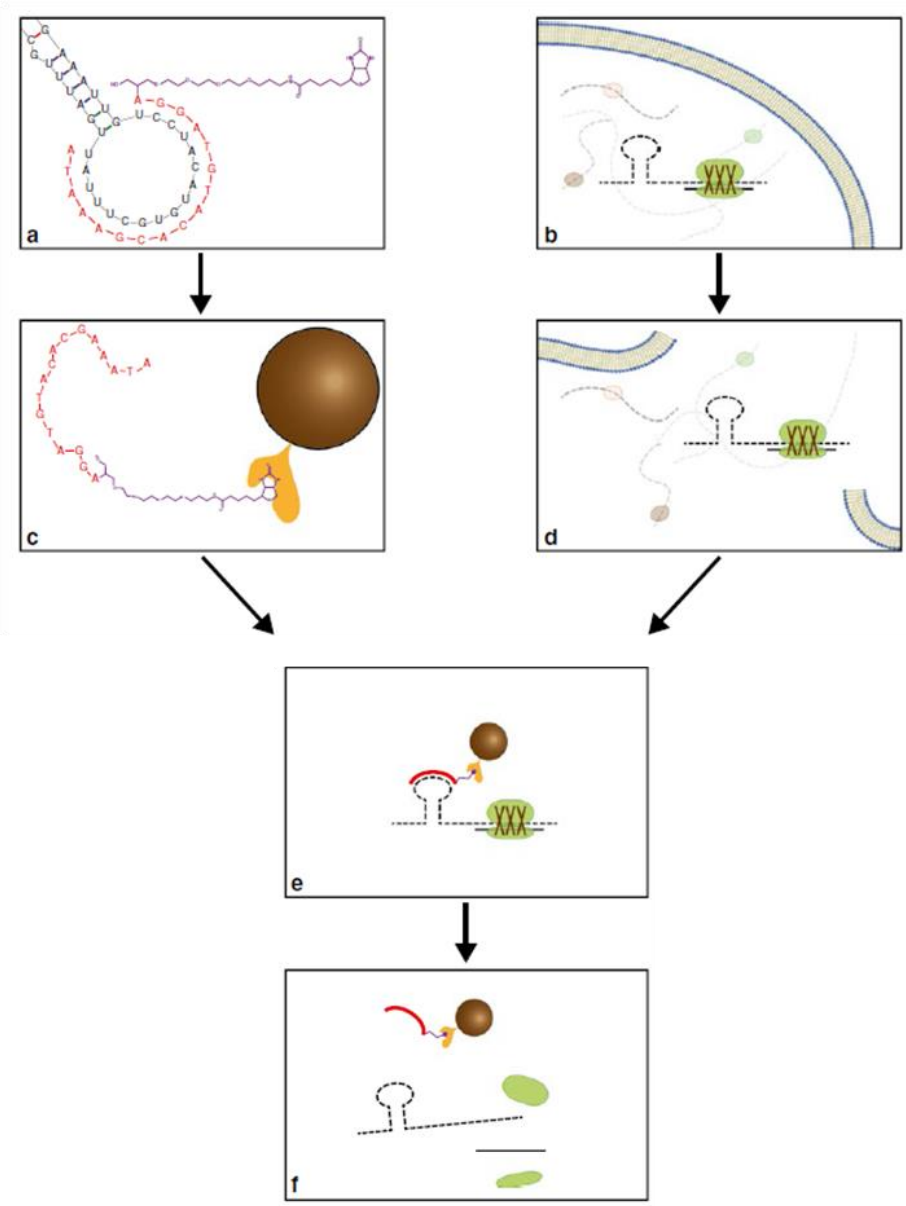


Figure 2.4 miR-CATCH strategy. **a**) synthesis of a biotin oligonucleotide targeting a single strand region of a target mRNA. **b**) Formaldehyde cross-linking step. **c**) oligonucleotide immobilization to magnetic streptavidine beads. **d**) cell lysis. **e**) the target mRNA is captured and purified. **f**) Formaldehyde cross-links are reversed (Vencken *et al*. 2015).

2.5 Luciferase assay

HeLa cells were plated in a 24-well plate (75.000 cells for each well) and transfected at 80% confluence using Lipofectamine LTX and Plus reagent (Life Technologies) with 15 ng of pGLO-3'UTR and 435 ng of miRNA-overexpressing plasmids. At 24 and 48h after transfection, cells were washed with PBS and lysed with Luciferase Assay Reagent (Promega). The lysates were used to measure Renilla and Firefly luciferase activity using

Dual-GLO Luciferase Assay System (Promega) with Infinite® M200 (Tecan) plate reader. The statistical significance was determined using Student's t-test comparison. Data were considered significant when $p<0,05$.

2.6 Western blotting and ELISA assay

HeLa cells were seeded in a 6-well plate and transfected using Lipofectamine LTX and Plus reagent (Life Technologies) or Lipofectamine 3000 with 2,2 µg of miRNA-overexpressing plasmids. At 48h after transfection, proteins were extracted with Radioimmunoprecipitation Assay Buffer (RIPA) supplemented with a protease inhibitor cocktail (Sigma-Aldrich) and analyzed by Western Blotting and ELISA assays. Proteins (20 µg) were separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nitrocellulose membrane by using iBlot® Dry Blotting System (Life Technologies®) at 20V for 7 minutes. Blots were first blocked with 5% non-fat powdered milk in PBS/Tween 0.1%, then probed overnight at 4 °C with mouse monoclonal anti-GRN (Abcam® no. ab55167, 1:500), rabbit polyclonal anti-Tau Dako (Agilent Technologies® A0024 , 1:1000) and rabbit polyclonal anti-HPRT (FL-218) (Santa Cruz Biotechnology® no. sc-20975, 1:500). Membranes were subsequently washed and incubated with anti-Mouse IgG and Anti-Rabbit IgG secondary antibodies (1:5000). Membranes were processed by ECL plus detection kit (Amersham®) according to manufacturer's instructions. Densitometric analysis was performed using ImageJ program.

Equal amount of proteins (1,5 µg) was used to perform ELISA assay (Adipogen®). Standards and samples were pipetted into the wells of a 96-well plate for binding to the pre-coated polyclonal antibody specific for progranulin. Washing steps were performed to remove unbound samples. Progranulin was recognized by the addition of a biotinylated polyclonal antibody and streptavidin labeled with HRP. After final washes, peroxidase activity was quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB) and the intensity of the color reaction. The intensity was directly proportional to the concentration of progranulin in the samples, measured at 450 nm. The quantification of progranulin in the analyzed samples was calculated with the use of a standard curve.

2.7 Quantitative Real Time-PCR

Total RNA was extracted from transfected or non transfected cells using TRIzol reagent (Invitrogen®), according to the manufacturer's instruction. For the quantification of GRN and MAPT transcripts from total RNA or polysomal/subpolysomal fractions, cDNA was synthesized by retrotranscription using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific®) with oligo (dT) primers, according to the manufacturer's protocol. Real-Time PCR (RT-qPCR) was performed using Kapa SYBR fast qPCR master mix (Kapa Biosystem®) and specific primers reported in **table 2.7**. PCR reaction (10 µl) contains 0,3 µM of each primer, 5 µl of master mix and 10 ng of cDNA. The expression of GRN and MAPT mRNAs was normalized with expression of different reference genes: glyceraldehydes-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase (HPRT1), succinate dehydrogenase complex subunit A (SDHA).

TaqMan MicroRNA Reverse Transcription kit (Life Technologies®) was used for miRNAs quantification. Total RNA (10 ng) was retro-transcribed using miRNA-specific stem–loop primers: hsa-miR-659-3p, hsa-miR-181a-5p, hsa-miR-615-5p, has-miR-608, has-miR-939-5p and endogenous controls small nucleolar RNAs (RNU44, RNU48 and RNU6). The PCR reaction (20 µl), contains 1.33 µl of cDNA, 10 µl of TaqMan 2X Universal PCR Master Mix, 1 µl of TaqMan MicroRNA Assay (20X) containing specific probes for miRNAs of interest. The reaction was incubated at 95°C for 10 minutes, then at 95°C for 15 s and 60°C for 60 s for 40 cycles. The relative expression of mRNAs and miRNAs was calculated by using the comparative Ct method. Data were expressed as fold-change relative to the mean of endogenous controls. The statistical significance was determined using Student's t-test comparison. Data were considered significant when $p<0,05$.

Table 2.7

Real Time PCR PRIMERS	SEQUENCES
Primer Forward GRN	5'-TTCTGGACAAATGGCCCAC-3'
Primer Reverse GRN	5'-ACCCACGGAGTTGTTACCTG-3'
MAPT Forward	5'-ACATCCATCATAAACCAGGAGGT-3'
MAPT Reverse	5'-TGTCTTGGCTTTGGCGTTCT-3'
HPRT I Forward	5'-TGACACTGGCAAAACAATGCA-3'
HPRT I Reverse	5'-GGTCCTTTTCACCAGCAAGCT-3'
SDHA Forward	5'-TGGGAACAAGAGGGCATCTG-3'
SDHA Reverse	5'-CCACCACTGCATCAAATTCATG-3'
GAPDH Forward	5'-TCTCCTCTGACTTCAACAGC-3
GAPDH Reverse	5'-CGTTGTCATACCAGGAAATGA-3'

2.8 Polysomal analysis

KELLY cells ($2x10^6$) were transfected in culture dishes (100mmx20mm, Corning®) with Lipofectamine 3000 (Life Technologies®). In every experiment two culture dishes were transfected with each miRNA over-expressing plasmid or empty control. After 48h the cells were incubated with cycloheximide to a final concentration of 100 μg/ml at 37°C for 3 minutes. The plates were subsequently kept on ice, the media was removed and the cells were washed with cold phosphate buffer saline (PBS) with cycloheximide 0.01 mg/ml. Cells were scraped directly on the plate with 300 μl cold lysis buffer [10 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris–HCl pH 7,5, 1% Triton X-100, 1% sodium deoxycholate, 0.2 U/ml RNase inhibitor, 1 U/μl DNase I, 1 mM dithiothreitol and 0.1 mg/ml cycloheximide], scraped and transferred to a eppendorf tube. The extracts were centrifuged for 5 min at 13000 rpm at 4°C. The supernatants were stored at -80°C and subsequently loaded on sucrose gradient containing 30 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, and centrifuged in an ultracentrifuge on a swinging rotor for 100 min at 180000 *g* at 4°C. Fractions (polysomal and sub-polysomal) were collected monitoring the absorbance at 254 nm and stored at -80°C. The fractions were treated with 0.1 mg/ml proteinase K for2 hours at 37°C. After phenol–chloroform extraction and isopropanol precipitation, polysomal and sub-polysomal RNAs were resuspended in 30 μl of RNAse free water and then used for Real Time PCR analysis.

CHAPTER THREE: IDENTIFICATION OF miRNAs INVOLVED IN THE POST-TRANSCRIPTIONAL REGULATION OF *GRN* EXPRESSION

3.1 Introduction

So far 69 different *GRN* mutations have been discovered as reported by the Alzheimer Disease and Frontotemporal Dementia Mutation Database (http://www.molgen.ua.ac.be/FTDmutations/) (Cruts *et al*. 2012). Accumulating evidences have demonstrated that cases of FTD caused by mutations in *GRN* lead usually to the degradation of the mutant RNA by nonsense-mediated decay with the loss of 50% functional GRN (Baker *et al*. 2006). Most of these mutations are frameshift, non-sense and splice-site mutations that cause a haploinsufficiency pathogenic mechanism through the degradation of the mutant allele (Baker *et al*. 2006, Cruts *et al*. 2006). These processes lead to a general decrease in the amount of progranulin, as it is observed in the serum or in the cerebrospinal fluid (CSF) of patients with *GRN* mutations that show a ~30–50% of the normal level of progranulin (Van Damme *et al*. 2008). Decreased amount of progranulin can also be detected in plasma or whole blood of *GRN* mutations patients (Finch *et al*. 2009; Coppola *et al*. 2008). For this reason molecular interventions that allow the increase in the production of progranulin are important as potential therapeutic strategies (Jiao *et al*. 2010). One way to control the production of progranulin is by acting at the translational level of GRN mRNA, where RNA binding proteins and miRNAs can play an important and relevant role. Little is known about miRNA regulation of GRN mRNA, except for two studies that identified binding sites on GRN mRNA for miR-29b and miR-107 that are reported to be significantly down-regulated in brains of Alzheimer's disease patients (Hebért *et al*. 2008, Wang WX *et al.* 2008). miR-29b is a demonstrated regulator of progranulin through a binding site localized on the 3'UTR of GRN mRNA (Jiao *et al*. 2010), whereas miR-107 can regulate progranulin through sequences present in the open reading frame of GRN mRNA (Wang-Xia *et al*. 2010). In particular in the last paper the binding of miR-107 and miR-103 was considered, since these miRNAs belong to the same gene family (miR-103/107) and they present a similar sequence (Wang-Xia *et al*. 2010). The authors found proof that glucose metabolism pathways can recruit miR-103/107 to down-regulate GRN expression

(Wang-Xia *et al*. 2010). Later on it was demonstrated that this miRNA family regulates insulin sensitivity and its silencing leads to an improved glucose homeostasis (Trajkovski *et al.* 2011).

Another scenario in which the decrease of GRN expression below a critical threshold can cause the development of FTD is due to the presence of SNPs (Rademakers *et al*. 2008). The genetic variability localized at the same loci implicated in Mendelian pathogenic diseases predisposes to sporadic forms of the pathology (Rademakers *et al*. 2005, Brouwers *et al*. 2006). An interesting study found, through genetic association analysis that a common genetic variation localized on the 3'UTR of *GRN* (78 C>T, rs5848) represents a genetic risk factor for FTD (Rademakers *et al*. 2008). The SNP, with the presence of the T allele, is localized on the seed sequence of miR-659-3p, causing the formation of 3 additional base pairs at the 5' end of miR-659-3p, that trigger a stronger binding and a more efficient repression compared to the wild type C allele. Indeed brain extracts derived from rs5848 TT homozygous FTD patients showed lower progranulin amounts compared to CC carriers, as observed through western blot analyses, ELISA assay and immunohistochemistry. The study proposed that a homozygous state of the T allele at rs5848 represents a risk factor which combined with environmental or other genetic factors will determine the individuals that develop FTD (Rademakers *et al*. 2008).

3.2 Results

3.2.1 GRN mRNA as a direct target of putative microRNAs

In order to identify the key miRNAs that could regulate GRN mRNA expression, three different bionformatic software programs were used to predict the potential miRNAs binding on the GRN 3'UTR sequence. Predictive results derived from PITA (http://genie.weizmann.ac.il/), Targetscan (http://www.targetscan.org/) and Targetprofiler (http://mirna.imbb.forth.gr/Targetprofiler.html) were integrated to increase the specificity. Each database predicted binding sites for hundreds of miRNAs, including one for miR-939-5p and one for miR-615-5p on GRN 3'UTR that was predicted by all three programs. The results were further screened and miR-608, miR-939-5p, miR-615-5p and miR-659-3p were selected according to the best score in terms of free binding energy, site accessibility and seed type (**Figure 3.2.1.1**). A multiple alignment using GRN 3'UTR sequences of three different species (mouse, rat and human) demonstrated a relevant level of conserved regions that might represent or indicate possible regulatory sequences (**Figure 3.2.1.2**). As described in **Figure 3.2.1.1**, miR-659-3p presents only one binding site, whereas miR-939-5p and miR-615-5p show three recognition elements with different specificity and binding energy. miR-608 shows two binding sites that are partially overlapping with the binding recognized by miR-939-5p, since they have a similar seed region. Overall the less conserved regions on GRN 3'UTR seem to be regions without putative miRNA binding sites.

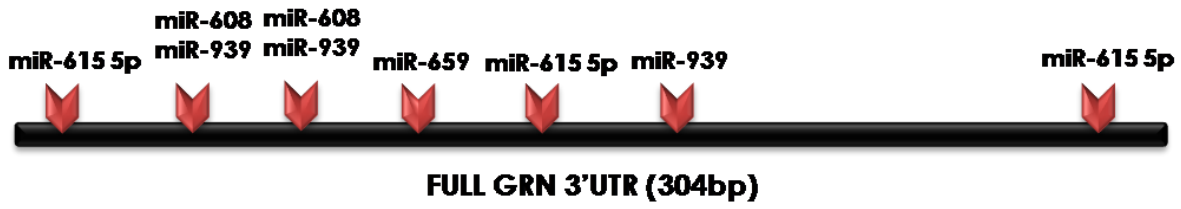


Figure 3.2.1.1 Scheme of the potential miRNAs binding site on GRN 3'UTR. Schematic representation of the putative miRNAs binding sites present on GRN 3'UTR according to the computational prediction of PITA, Targetscan and Targetprofiler.

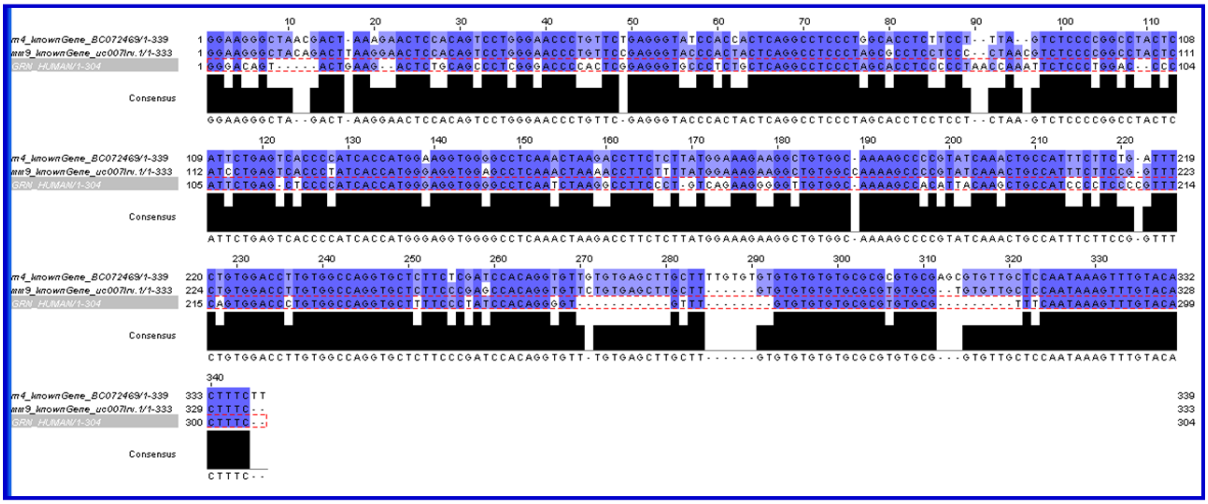


Figure 3.2.1.2 Conservation analysis of GRN 3'UTR between human, mouse and rat. Multiple alignment of GRN 3'UTR sequence between human, mouse and rat species performed through ClustalW and graphically represented with Jalview. Black squares below the alignment and dark blue color on the sequences indicate the 100% conservation between human, mouse and rat.

3.2.2 GRN 3'UTR Analysis of SNPs Located on miRNAs Binding Sites and Sequencing of GRN 3'UTR in Different Cell Lines

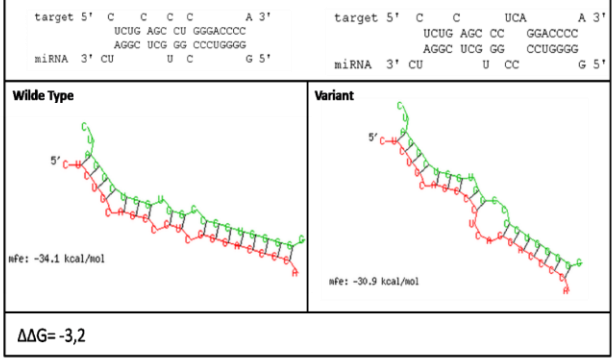
Neurodegenerative diseases are strongly influenced by the genetic variability present in the human population (Hulse and Cai, 2013; Lukiw, 2013). Indeed variants located in important regulatory regions could have important effects in different disorders. In the present study sixteen different SNPs were identified in the GRN 3'UTR using dpSNPs database. Six of these SNPs were found in miRNAs binding sites. RNAhybrid software was used in order to identify the capacity of these SNPs to alter the interaction between the predicted miRNAs and GRN 3'UTR. Indeed RNAhybrid computes in silico hybridization of the miRNA with the mRNA in presence of the common or variant allele. The common genetic variability could increase or decrease the interaction binding and this would be reflected in the alteration of the free binding energy (ΔG) calculated by RNAhybrid. The difference between the free binding energy of the common and variant allele can be computed as variation (ΔΔG) as reported by Saba and colleagues (Saba *et al*. 2014). ΔG values that are more negative correspond to higher stability of miRNA-mRNA interaction, whereas ΔG values that are more positive indicate a lower stability interaction. According to the method developed by Saba and coworkers, SNPs can be classified in three different groups based on

the ΔΔG values: (ΔΔG>0 kcal/mol) SNPs that increase miRNA binding, (ΔΔG<0 kcal/mol) SNPs that reduce miRNA binding and (ΔΔG=0 kcal/mol) neutral SNPs that have no effect on miRNA binding (Saba *et al.* 2014). In the **Figure 3.2.2a** are reported the comparisons between the wild type and the variant miRNA-GRN 3'UTR interactions and the computed ΔΔG value for each binding. Only two SNPs caused an enhanced miRNA binding, one is located on the I binding of miR-608 (**Figure 3.2.2a D)** and the other is present on the binding of miR-659-3p (**Figure 3.2.2a G)**. This variation overlapped miR-659-3p interaction was already reported by Rademakers and colleagues and represented a genetic risk factor for FTD (Rademakers *et al.* 2008). All the other SNPs overlapping miRNAs interaction regions induced a decreased miRNA binding, except for one SNP found on the II binding of miR-615-5p-5p that has no effect on the interaction, as represented in the **Figure 3.2.2a I.**

Target: GRN 3'UTR
miRNA: miR-615-5p I Binding Site

SNP: rs374114836, Alleles A/G

A

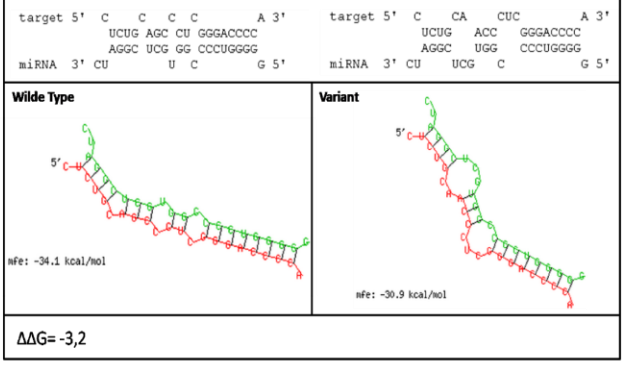


ΔΔG= -3,2

Target: GRN 3'UTR
miRNA: miR-615-5p I Binding Site

SNP: rs563619928, Alleles A/G

B

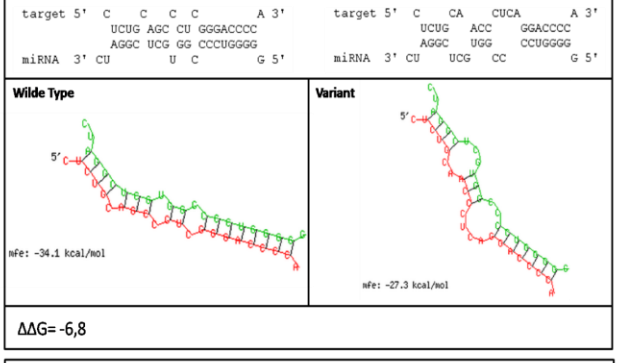


ΔΔG= -3,2

Target: GRN 3'UTR
miRNA: miR-615-5p I Binding Site

2 SNPs: rs374114836, Alleles A/G
rs563619928, Alleles A/G

C

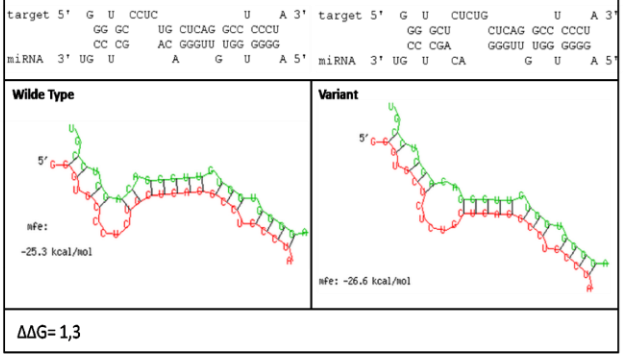


ΔΔG= -6,8

Target: GRN 3'UTR
miRNA: miR-608 I Binding Site

SNP: rs372352654, Alleles C/T

D

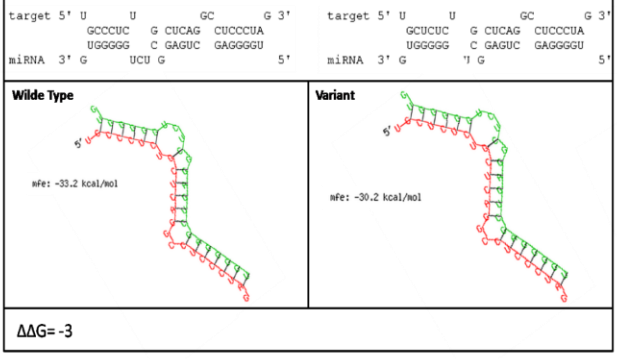


ΔΔG= 1,3

Target: GRN 3'UTR
miRNA: miR-939 I Binding Site

SNP: rs372352654, Alleles C/T

E

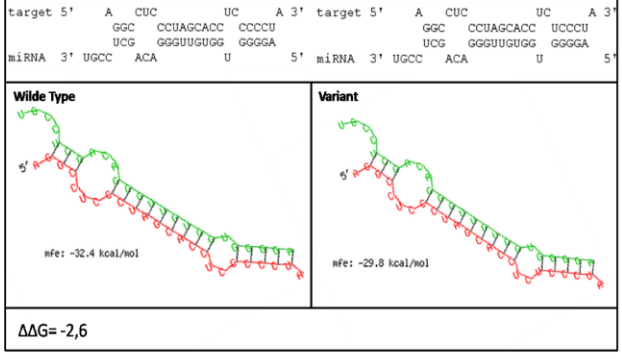


ΔΔG= -3

Target: GRN 3'UTR
miRNA: miR-608 II Binding Site

SNP: rs5848, Alleles C/T

F



ΔΔG= -2,6

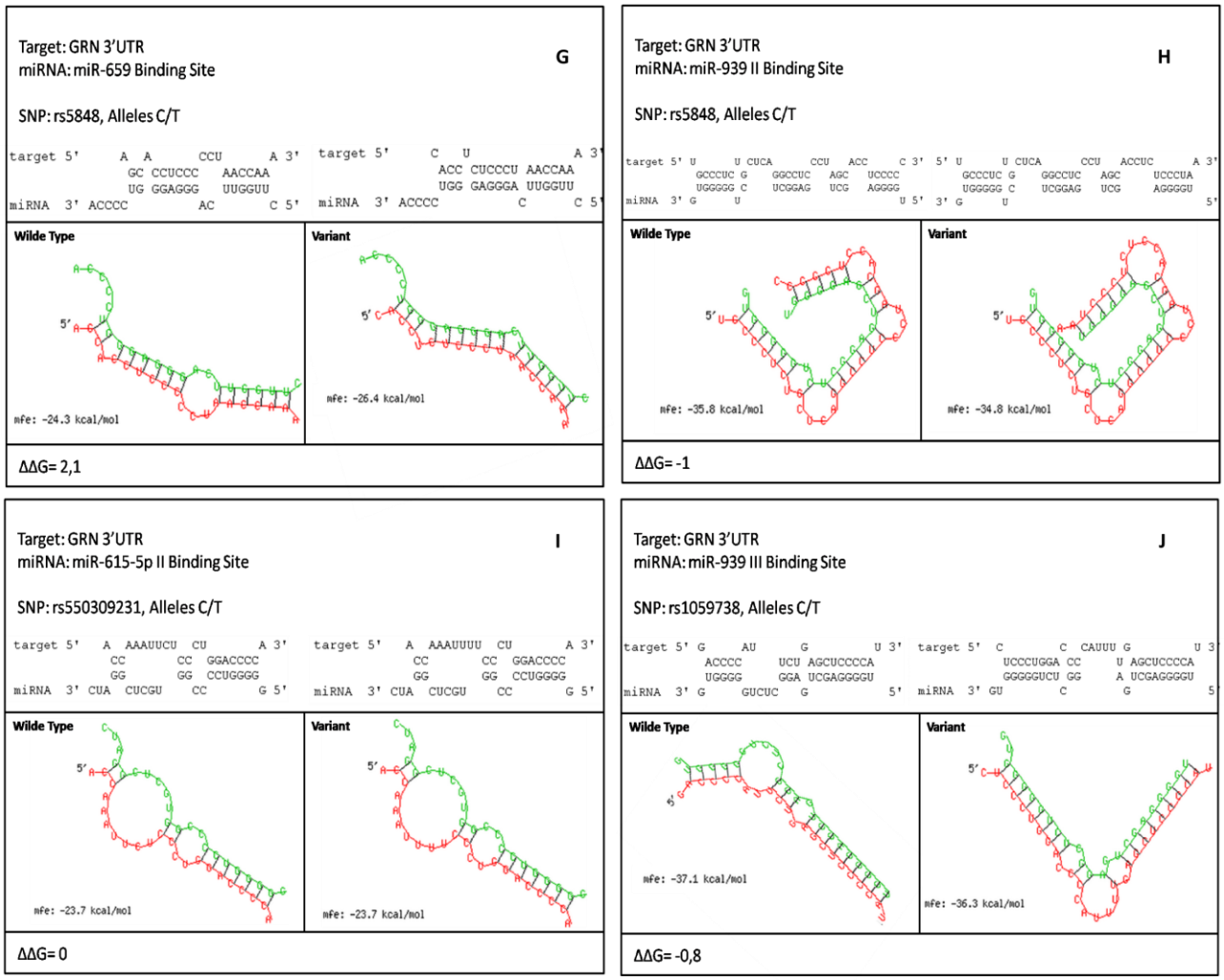


Figure 3.2.2a. SNPs overlapping miRNA binding sites analysis for GRN 3'UTR
Representation of the miRNA-GRN mRNA functional characterization technique following the method reported by Saba and colleagues (Saba *et al*. 2014).

GRN 3'UTR was further characterized through sequencing in all the cell lines used: HeLa, KELLY, SH-SY5Y and SK-N-BE. Interestingly there was a different presence of the alleles T or C on the SNP rs5848 found in FTLD patients by Rademakers and colleagues (Rademakers *et al.* 2008). As reported in the **Figure 3.2.2b** HeLa and SH-SY5Y cell lines are homozygous C-allele, while SK-N-BE cell line is heterozygous T/C allele, with a major prevalence of T allele and KELLY cell line is homozygous for T allele.

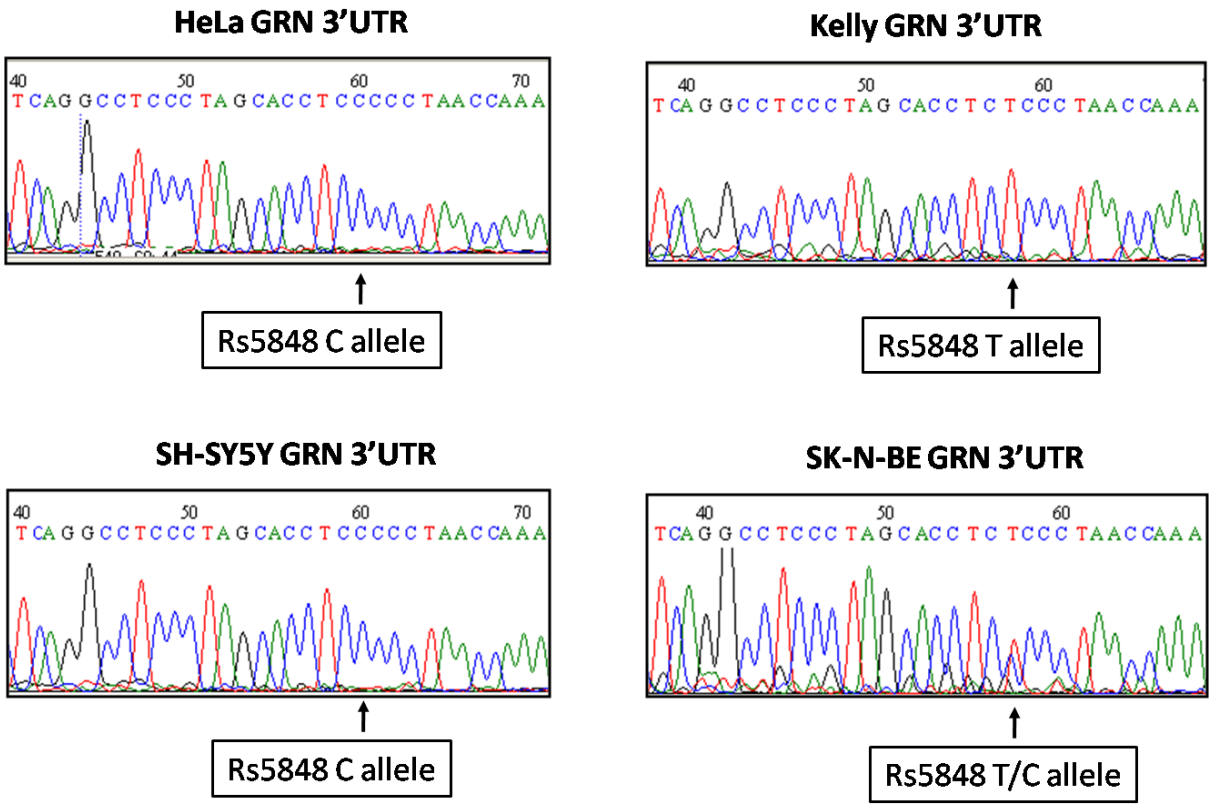


Figure 3.2.2b GRN 3'UTR sequencing in HeLa, KELLY, SH-SY5Y and SK-N-BE cell line. Representation of chromatograms of the *GRN* 3'UTR sequencing performed in different cell line. The images report the region of *GRN* 3'UTR characterized by the common genetic variant rs5848 (78 C>T).

3.2.3 Expression of GRN mRNA in different neuroblastoma cell lines

Neuroblastoma cell lines were used as a neuronal-like system to study the regulation of our genes of interest, GRN and MAPT in normal cell condition. RT-PCR was used to better analyze GRN mRNA expression in different neuroblastoma cell lines. Amplification of a 200 bp product, located on GRN mRNA between exon 1 and 4, was obtained with specific primers recognizing regions of exons junctions. In particular, forward primer binds to a region located between exon 1 and 2, while reverse primer recognizes a sequence located on exons 3 and 4.

The level of progranulin expression as mRNA was quite similar in all cell lines analyzed: IMR32, LA-N-1, LA-N-2, SK-N-MC, SK-N-AS, SK-N-DZ, SK-N-SH, SK-N-BE, CHP134, CHP212, SIMA, KELLY, NB-69, except for SIMA and IMR32 cell lines, where GRN mRNA seems to be expressed at a lower level. In order to investigate GRN mRNA post-transcriptional regulation, KELLY, SH-SY5Y (derived from SK-N-SH) and SK-NBE cell lines were selected since they show a meaningful level of GRN mRNA expression.

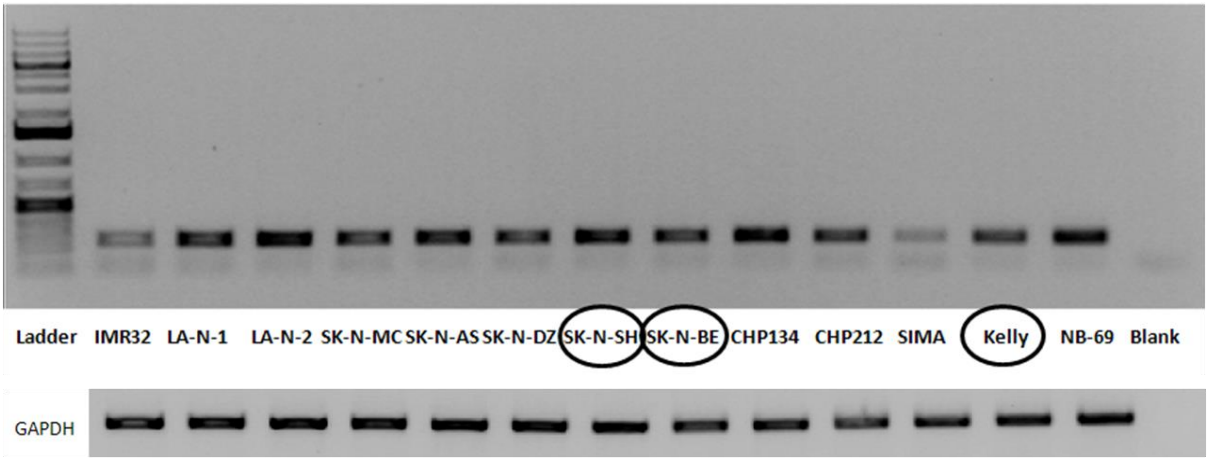


Figure 3.2.3 RT-PCR of GRN mRNA in different neuroblastoma cell lines. Image of an agarose gel showing amplification of 200 bp products of GRN mRNA in different neuroblastoma cell lines. Black circles represent the selected neuroblastoma cell lines.

3.2.4 miRNAs Basal Expression in different cell lines

Using TaqMan quantitative RT-PCR (qRT-PCR), the endogenous expression of miR-939-5p, miR-608, miR-659-3p and miR-615-5p was analyzed in four cell lines: HeLa, KELLY, SK-N-BE and SH-SY5Y. All C_T levels were normalized against snRNU6 to calculate the values $2^{-\Delta Ct}$. In **Figure 3.2.4** we can observe that miR-939-5p, in all the different cell lines, is expressed at higher level compared to the other selected miRNAs. Indeed, miR-659-3p is expressed at low level, mainly in SH-SY5Y and SK-NBE cell lines, whereas miR-615-5p and miR-608 are present at minimal level or totally absent in the analyzed cells.

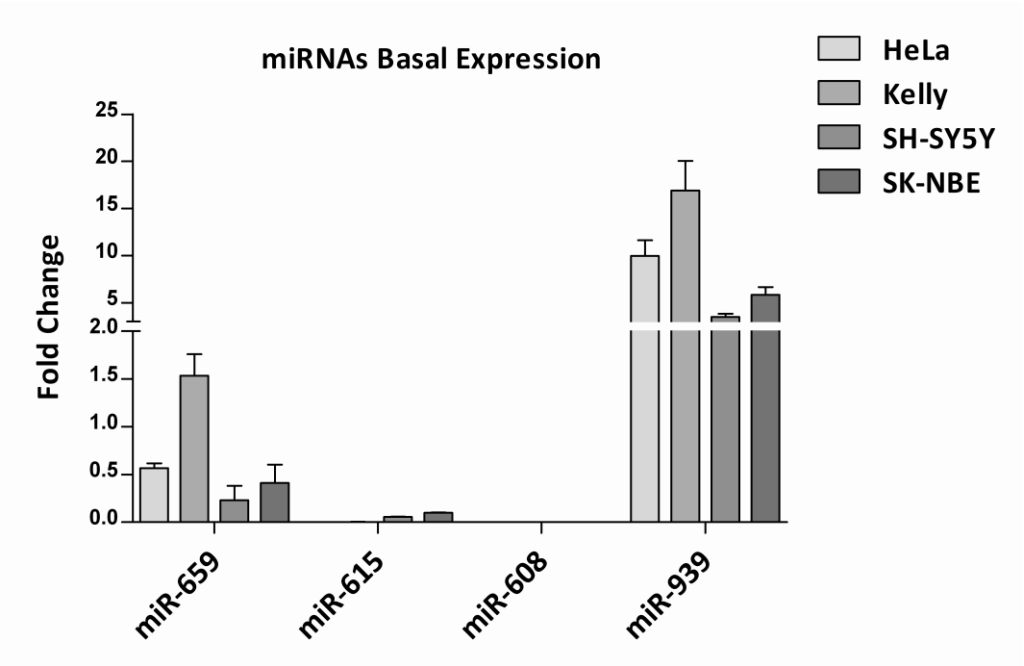


Figure 3.2.4. miRNAs basal expression levels in HeLa, KELLY, SH-SY5Y and SK-NBE cell lines. Representation of fold change ($2^{-\Delta Ct}$) values, derived from the normalization with RNU6 C_T levels. qRT-PCR image is representative of one biological experiment with three technical replicates (Mean ±SD).

3.2.5 GRN microRNAs Capture Affinity Technology (miR-CATCH)

miRNA targets have been predicted through different bioinformatic programs that are based on the free binding energy, site accessibility, seed type and seed sequence conservation. However, the miRNAs target recognition mechanism remains challenging, due to the high false-positive rates and to the fact that computational analysis relies on different algorithms that yield different results. For this reason it became clear the necessity to use a biochemical strategy based on physiological interactions between miRNPs and their specific target mRNAs to reliably identify subset of miRNAs targeting GRN mRNA. The microRNA Capture Affinity Technology (miR-CATCH) developed by Prof. Catherine M. Greene and her team (Hassan *et al*. 2013, Vencken *et al*. 2015), allows to selectively identify miRNAs binding to a specific mRNA under different conditions, eliminating false positive mRNA:miRNA interactions that can be predicted *in silico*. This technique offers the possibility to identify miRNAs that bind to the entire mRNA transcripts, therefore it is not limited to the 3'UTR region. It is a novel way to bypass or complement the initial use of bioinformatic predictions. I moved to the laboratory of Prof. Catherine M. Greene, where I worked for four months to learn and applied this technique in collaboration with Dr. Sebastian Vencken*. Firstly, we used KELLY cells for the validation of the isolation method and the efficacy of the miR-CATCH protocol specificity. Then, the miR-CATCH strategy was used to investigate the regulation played by miRNAs of GRN mRNA in the selected neuroblastoma cell lines: KELLY, SH-SY5Y and SK-N-BE.

*Optimization of the miR-CATCH technique for the isolation of GRN and MAPT mRNAs was done in collaboration with Dr. Sebastian Vencken, at the laboratory of Respiratory Research at the Royal College of Surgeons in Ireland, Beaumont Hospital.

3.2.5.1 Biotinylated DNA Oligonucleotides Design

The secondary structure of GRN mRNA was modeled using M-Fold web server (http://mfold.bioinfo.rpi.edu). Two of the most thermodynamically stable structures were elaborated and analyzed through the UGENE program (http://ugene.unipro.ru/) for a better identification of single strand regions present with the highest probability in the GRN mRNA structures. The exposed single strand region localized between bases 1276 and 1303 of the GRN transcript was used as a target sequence to design the specific DNA capture oligonucleotide, that should have a low hairpin melting temperature and a high hybrid melting temperature to hybridize with the single strand region. The anti-sense capture DNA oligonucleotide was biotinylated at the 5' end and was designed specifically against the single strand region identified on GRN mRNA (5'-TCT TCA AGG CTT GTG GGT CTG GCA GG-3'), as represented in the **Figure 3.2.5.1 and Table 3.2.5.1a**. The region of 27 bp was analyzed with Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi), obtaining an exclusive 100% of total coverage to GRN mRNA. However BLAST showed a 15 bp similarity with BDNF-Antisense RNA (BDNF-AS) and an 18 bp similarity with lymphocyte-specific protein tyrosine kinase (LCK). BLAST was also used to identify a scrambled sequence not specific for any transcripts in order to design a DNA scrambled 5' terminally biotinylated oligonucleotide (5'-ATA TAT TAG ATT GCG TAT AAT TAG G-3') as a non specific control of the miR-CATCH. Finally, in order to control the sequence specificity of the GRN capture oligonucleotide, a mismatch DNA capture oligonucleotide biotinylated at the 5'end was designed, changing 3 nucleotides (underlined altered bases) from the original sequence (5'-TCT TCA AGG CTT <u>ACA</u> GGT CTG GCAGG-3'), as reported in the **Table 3.2.5.1b**.

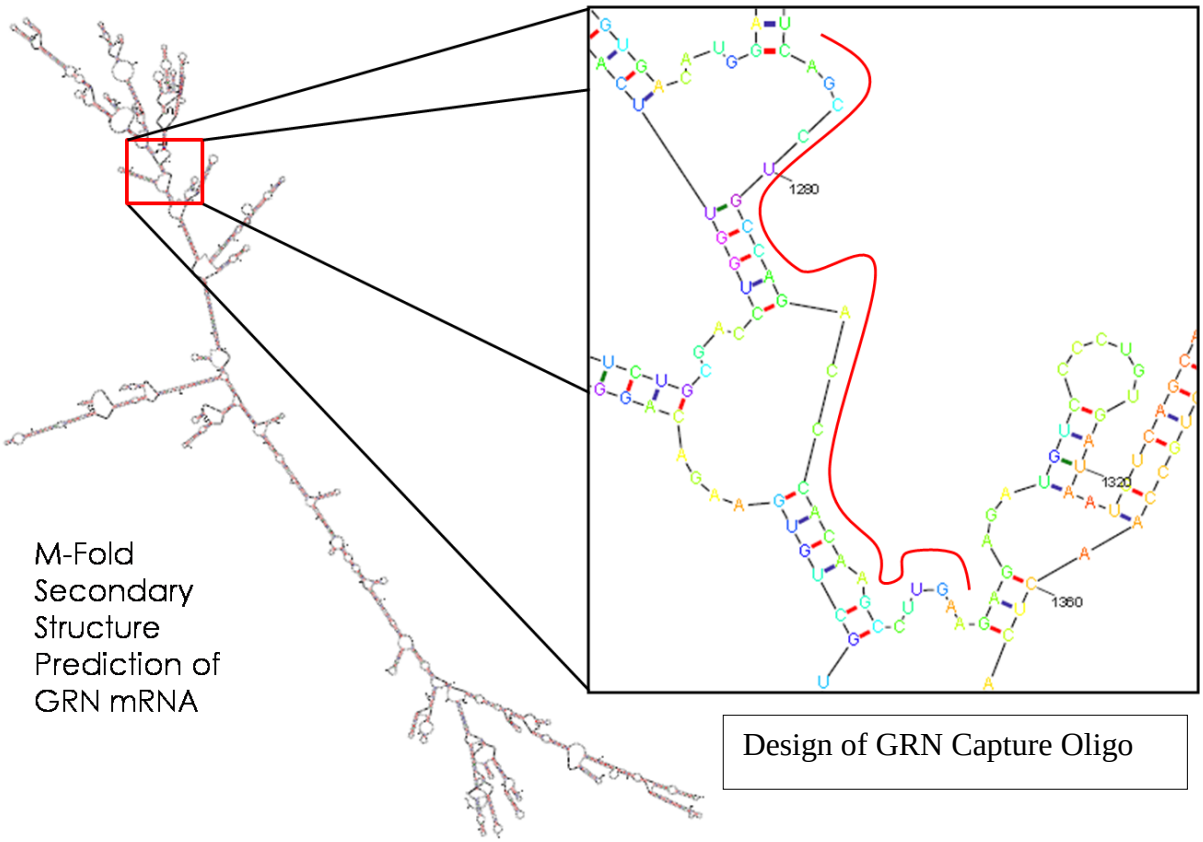


Figure 3.2.5.1 Design of GRN mRNA Capture Oligonucleotide. Secondary structure of GRN mRNA was elaborated through M-Fold. The exposed single strand region located between bases 1276 and 1303 of the transcript was used as a target.

SINGLE STRAND REGION :	5'-AGCCTGCCAGACCCACAAGCCTTGAAGA-3'
REVERSE AND COMPLEMENT SEQUENCE:	……. 5'-TCTTCAAGGCTTGTGGGTCTGGCAGGCT-3'
OLIGO SEQUENCE:	Biotin 5'-TCT TCA AGG CTT GTG GGT CTG GCA GG-3'
LENGTH:	26
GC CONTENT:	57,7%
MELTING TEMPERATURE:	79,8°C
HAIRPINS TM:	36,5°C 34,5°C 29,4°C 31,6°C 31,5°C
HOMO DIMER ANALYSIS:	≥-5,5 Kcal/mole

Table 3.2.5.1a Sequence and characteristics of the designed GRN mRNA Capture Oligonucleotide

OLIGO SEQUENCE:	5’-TCT TCA AGG CTT GTG GGT CTG GCA GG-3’
MISMATCH OLIGO SEQUENCE:	Biotin 5’-TCT TCA AGG CTT ACA GGT CTG GCAGG-3’
LENGTH	26
GC CONTENT	53,8%
MELT TEMP	76,5°C
Hairpins Tm:	39,4-36,5-29,9-31,6-30,6

Table 3.2.5.1b Sequence and characteristics of the designed GRN mRNA Mismatch Capture Oligonucleotide

3.2.5.2 Validation of GRN mRNA:miRNA Isolation

In order to validate the isolation method and the efficacy of the miR-CATCH technique to enrich GRN mRNA in the capture sample, we performed the first experiments in KELLY cells. Levels of GRN mRNA in the capture samples obtained by qRT-PCR were compared to the levels obtained from the same cell lysates that did not undergo the capture procedure. After normalization with GAPDH and using the $2^{-\Delta\Delta Ct}$ method, we obtained an enrichment of 17,55 fold compared to the total RNA extracted from the same lysate of KELLY cells, as reported in the **Figure 3.2.5.2a**. Then, to investigate the efficiency of capture oligonucleotide sequence in the level of enrichment, another miR-CACTH experiment was performed using the capture oligonucleotide and the mismatch capture oligo containing 3 different bases. There was a GRN mRNA enrichment of 33,3 fold obtained with the capture oligonucleotide and reduction of 18,5 units obtained with the mismatch oligonucleotide. Indeed the mismatch capture oligonucleotide cause an enrichment of 14,7 fold compared to the total RNA, as reported in the **Figure3.2.5.2b**. The reduction in the enrichment level demonstrated the importance of the sequence specificity of the capture oligonucleotide used to specifically pull down GRN mRNA. As reported previously the sequence of GRN capture oligonucleotide showed complementarity with other two transcripts: BDNF-Antisense RNA (BDNF-AS) and a lymphocyte-specific protein tyrosine kinase (LCK). Specific primers were designed for the detection of these two off targets in the capture samples. However the qRT-PCR did not show enrichment for these two additional transcripts in the capture samples compared to the total RNA (data not shown).

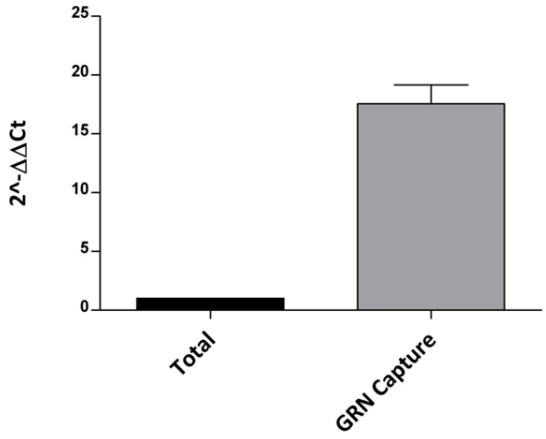


Figure 3.2.5.2a GRN mRNA enrichment. Representation of the real time PCR results showing the fold enrichment level ($2^{-\Delta\Delta Ct}$) obtained with GRN capture oligonucleotide compared to the total RNA that did not undergo the miR-CATCH protocol. Expression of GRN mRNA was normalized against GAPDH expression. Representative data of one biological experiment and three technical replicates.

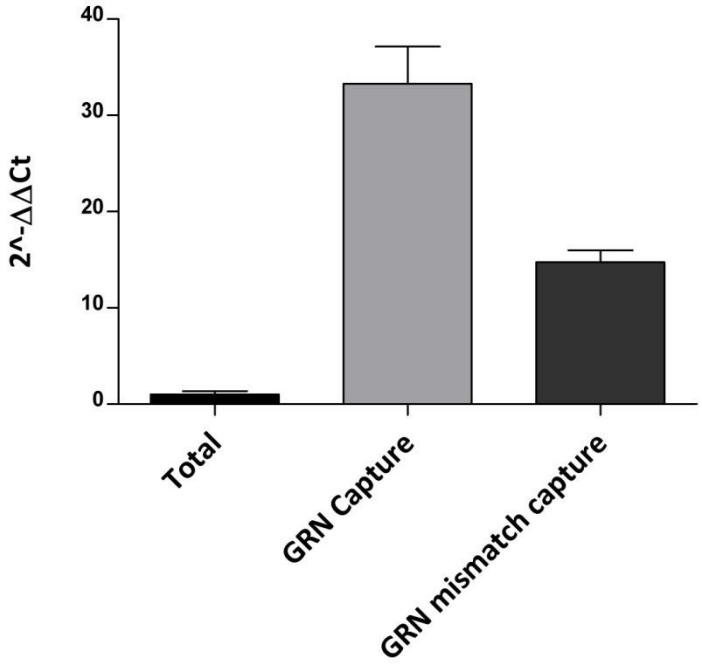


Figure3.2.5.2b. Validation of GRN mRNA isolation. Representation of the qRT-PCR data, showing the fold enrichment level ($2^{-\Delta\Delta Ct}$) obtained with GRN capture oligonucleotide compared to the total RNA that did not undergo the miR-CATCH protocol and to the level obtained with a biotinylated mismatch oligonucleotide. Expression of GRN mRNA was normalized against GAPDH expression. Representative data of one biological experiment with three technical replicates.

3.2.5.3 GRN mRNA Enrichment in KELLY, SK-N-BE and SH-SY5Y cell lines

Following the validation of the miR-CATCH protocol in the KELLY cell line, the pull-down method was performed in biological triplicates in selected neuroblastoma cell lines. "Scrambled" samples were obtained from the same cell lysate of each biological experiment, using a scrambled capture oligonucleotide biotinylated at the 5'end for the miR-CATCH strategy. Using qRT-PCR, the level of GRN mRNA enrichment was assayed individually in each biological experiment and compared to the scrambled control through a $2^{-\Delta\Delta Ct}$ method. In particular 47 fold enrichment of GRN mRNA was obtained in the KELLY cell line, whereas 66 fold enrichment was found in SH-SY5Y cell line and 23 fold enrichment for SK-N-BE cell line, as reported in the **Figure 3.2.5.3.** Data represents the mean of three biological replicates.

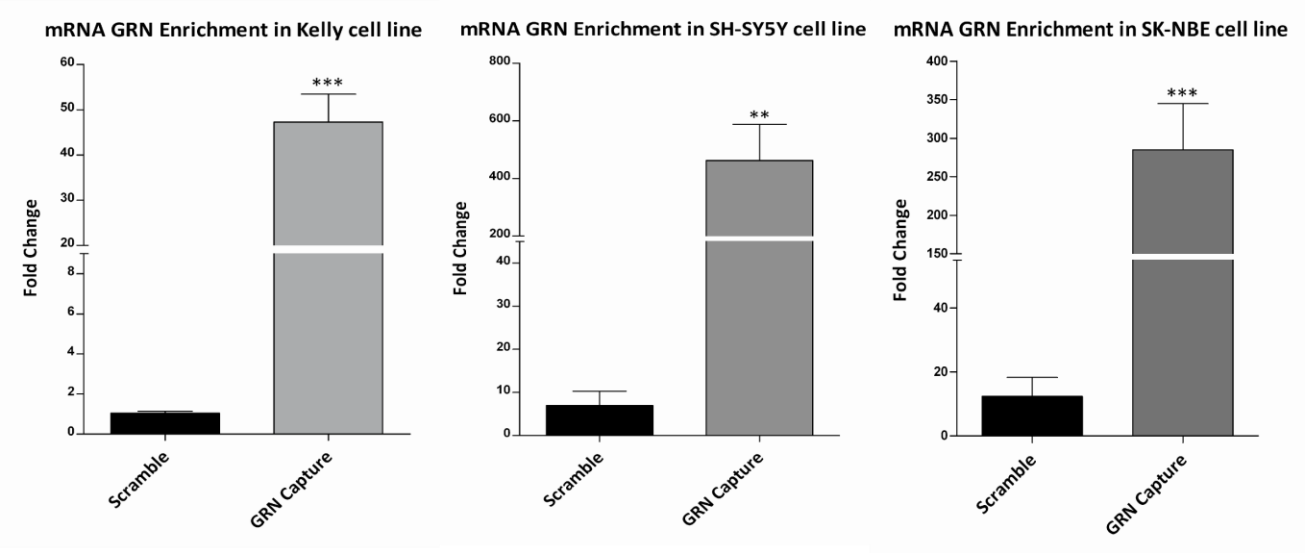


Figure 3.2.5.3 GRN mRNA Enrichment in different neuroblastoma cell lines. Representation of the fold enrichment ($2^{-\Delta\Delta Ct}$) values of GRN mRNA compared to a specific scrambled control. Data were normalized with GAPDH expression. Mean ± SEM of three biological replicates is shown in the figure (** p<0,001; *** p<0,0001 vs. scrambled control).

3.2.5.4 Selected miRNA Profiling on Capture Samples in KELLY, SH-SY5Y and SK-N-BE cell lines

The enrichment of putative miRNAs of our interest (miR-659-3p, miR-939-5p, miR-608 and miR-615-5p), predicted through the use of bioinformatic softwares, was detected by TaqMan qRT-PCR. The level of fold enrichment was calculated comparing the capture samples with the specific scrambled controls using the $2^{-\Delta Ct}$ method. The figures represent the mean level of the specific miRNA enrichment derived from the biological triplicates for each different cell line. In **Figure 3.2.5.4.a** and **3.2.5.4.b** are reported the specific level of enrichment for each miRNA. miR-659-3p shows an enrichment of 15 times in GRN capture samples derived from the KELLY cell line (**Figure 3.2.5.4.a A**), 1959 times and 259 times respectively in GRN capture samples derived from SH-SY5Y and SK-N-BE cell lines (**Figure 3.2.5.4.b A and B**). Whereas miR-939-5p was found weakly enriched (1,6 times) in GRN capture samples derived from the KELLY cell line (**Figure 3.2.5.4.a B**), slightly enriched (1,5 times) in GRN capture samples of SK-N-BE cells and not enriched in GRN capture samples of SH-SY5Y cell line (**Figure 3.2.5.4.b A and B)**. miR-608 and miR-615-5p were also quantified by TaqMan qRT-PCR, however these miRNAs were not found enriched in capture samples, probably due to their lack of expression in all the analyzed neuroblastoma cell lines at physiological conditions (data not shown). The expression of miR-323 was used as a negative control in the KELLY cell line. miR-323 was not enriched in the capture samples compared to scrambled controls (**Figure 3.2.5.4.a C**).

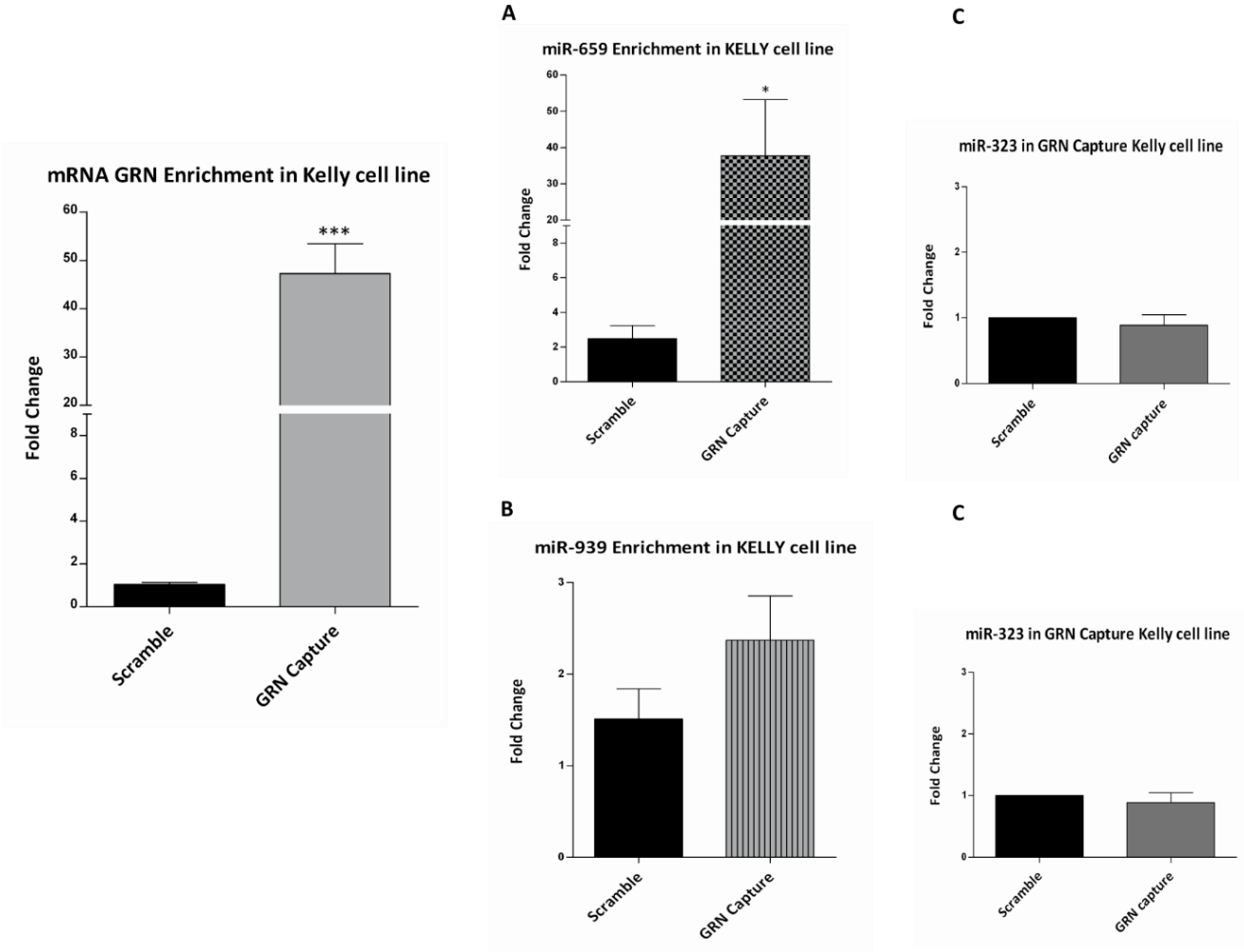


Figure 3.2.5.4.a. Selected miRNA enrichment in KELLY cell line. Representation of miR-659-3p **A)** and miR-939-5p **B)** enrichment in GRN capture samples compared to scrambled controls. Graphs on the right part of the figure **C)** represent the negative control miR-323 that shows no enrichment in the capture samples. miRNAs expression was represented as fold enrichment value ($2^{-\Delta Ct}$).
A) and **B)** Mean±SEM of three different biological experiments (*P<0,05; *** P<0,0001). **C)** Representative figure of two biological samples with three technical replicates.

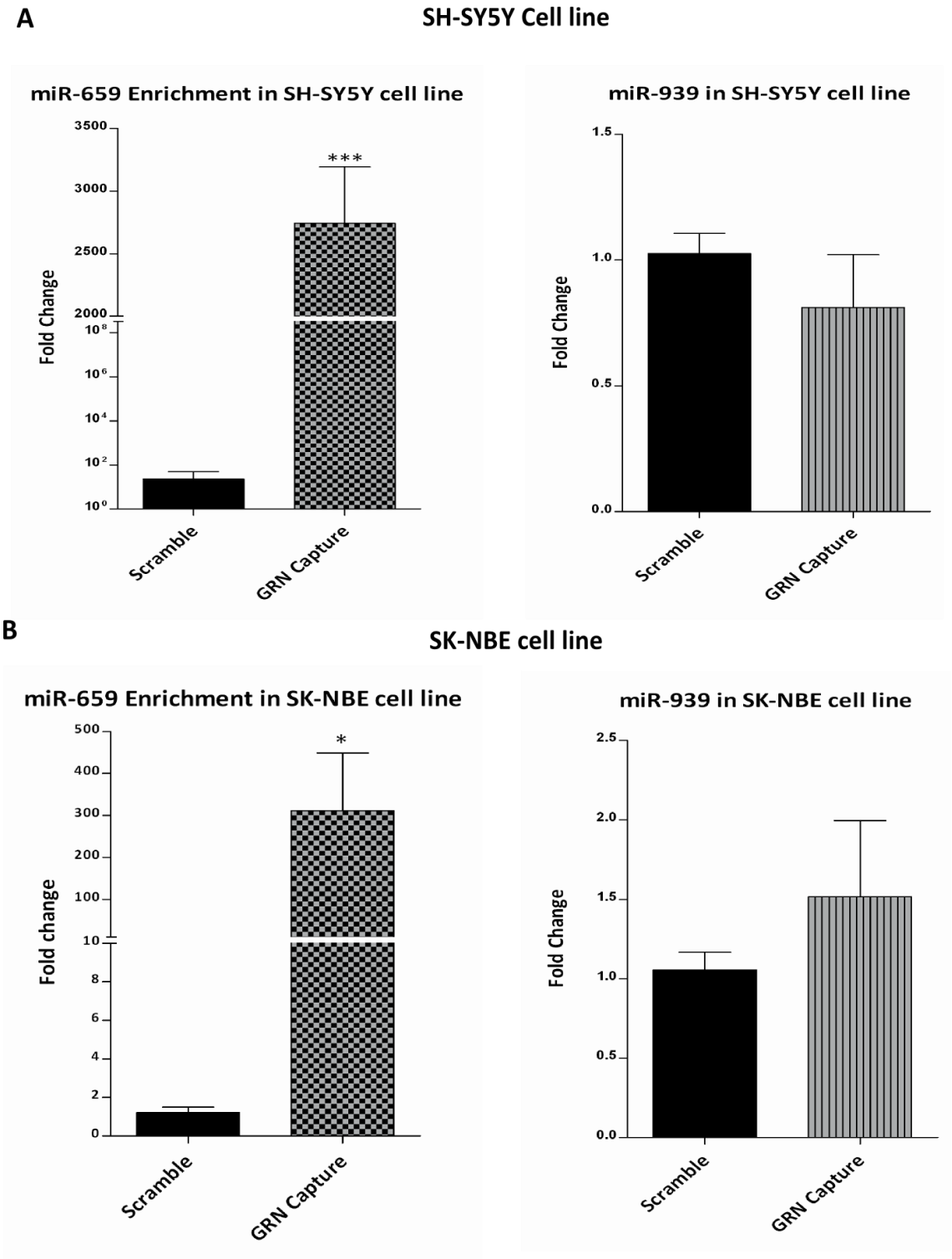


Figure 3.2.5.4.b Selected miRNAs enrichment in SH-SY5Y and SK-NBE cell lines. A) Representation of miR-659-3p and miR-939-5p enrichment found in capture samples compared to scrambled controls in SH-SY5Y samples. **B)** Graphs representing the enrichment of miR-659-3p and miR-939-5p compared to the scrambled controls in SK-NBE cell line. miRNA expression was represented as fold enrichment value ($2^{-\Delta Ct}$).
Mean ± SEM of three different biological experiments (* P < 0,05; *** P < 0,0001).

3.2.6.1 Luciferase Assays using reporter vectors of the full GRN 3'UTR in HeLa cell line

Following the results obtained with miR-CATCH strategy, the putative miRNA's regulation on GRN 3'UTR was further investigated using luciferase activity assays in HeLa cell line. HeLa cell line is a reliable cell system, easy to handle and useful to explore with high efficiency the regulation of our miRNAs of interest by co-transfection experiments. According to our bioinformatic prediction, miR-181a does not have binding sites localized on GRN 3'UTR and for this reason it was selected as a negative control. HeLa cells were co-transfected with reporter vectors containing the full length or different regions of GRN 3'UTR and miRNA over-expressing plasmids.

3.2.6.1.1 Analysis of the miRNAs Overexpression in HeLa cell line

Firstly, was checked the specific miRNA's over-expression, during the luciferase analysis performed at 24h and 48h after transfection, the RNA was extracted and miRNAs quantified by qRT-PCR based on TaqMan assay. All the C_T levels were normalized to RNU6, to calculate the fold change with the $2^{-\Delta Ct}$ method. In **Figure 3.2.6.1.1a** the basal endogenous level of expression of selected miRNAs (light green) is compared to the level of miRNAs over-expressed from plasmids (dark green). Moreover the ΔΔCt values were calculated using as normalizers the miRNA levels measured after transfection of backbone empty plasmid, utilized for miRNA cloning. These fold changes are reported in the **Figure 3.2.6.1.1b.**

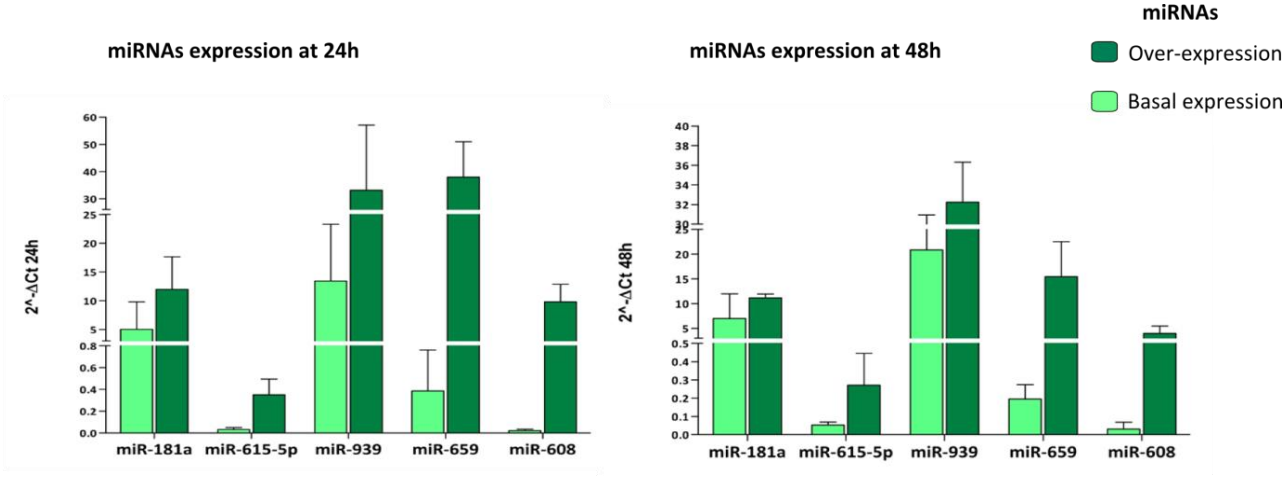


Figure 3.2.6.1.1a Basal and Over-expression levels of selected miRNAs in HeLa cell line. TaqMan real time PCR was performed on the RNA extracted from parallel samples derived

from the luciferase analysis at 24h and 48h. Ct levels are normalized against RNU6 to calculate the fold change with the $2^{-\Delta Ct}$ method. Data are obtained from biological duplicates and technical triplicates (Mean ±SEM).

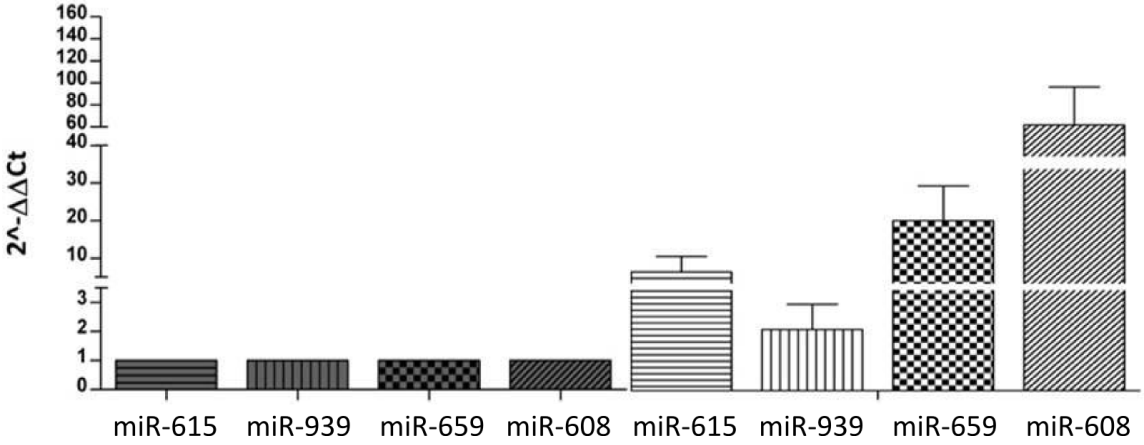


Figure 3.2.6.1.1b Over-expression levels of selected miRNAs in HeLa cell line. Representation of the $2^{-\Delta\Delta Ct}$ values obtained through the comparison between the over-expression of miRNAs and the endogenous basal level of expression in HeLa cell line. Data represent the summary of biological duplicates and technical triplicates (Mean ±SEM).

3.2.6.1.2 Validation of mirtron-939 over-expression

The human precursor region encoding miR-939-5p, overlaps with a 5' splice site as reported in the **Figure 3.2.6.1.2a** taken from Ensembl Genome Browser. The stem-loop precursor region of miR-939-5p has the typical hairpin-end structure of mammalian mirtrons composed by GUGGG nucleotides, proposed by Berezikov and colleagues (Berezikov et al. 2007). miR-939-5p could be considered as a 3'tailed mirtron in which the spliceosome and the microprocessor complex compete for the same region, leading to a spliced product or the generation of a mature miRNA. The precursor region of miR-939-5p cloned inside the over-expressing plasmid for miRNAs, under the control of U1 promoter and U1 terminator, contains two exons with three entire introns as reported in the **Figure 3.2.6.1.2b**. The precursor region of miR-939-5p that is unspliced has a length of 465 bp, whereas the spliced region will be of 272 bp. In order to validate if splicing competes with the miR-939-5p biogenesis and maturation, RT-PCR was performed using RNA extracted from cells that have been transfected with the over-expressing plasmid for miR-939-5p. Primers used for the cloning of miR-939-5p precursor region were used to amplify the region of interest. Samples were loaded on 2% agarose gel. **Figure 3.2.6.1.2c** depicts the

results, showing a stronger production of the unspliced product of 465 bp compared to the spliced product of 272 bp represented by a faint band. This result demonstrated a major recruitment of the microprocessor complex on the unspliced precursor region of miR-939-5p. The production of miR-939-5p seems to be preferred compared to the splicing of that region that would lead otherwise to the disruption of the precursor sequence for miR-939-5p.

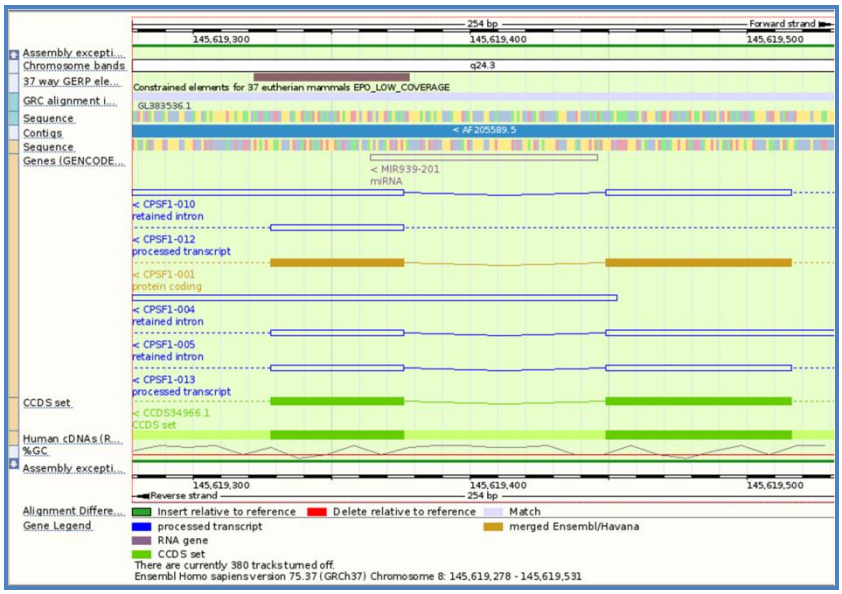


Figure 3.2.6.1.2a Ensembl Genome Browser representation of miR-939-5p precursor region.

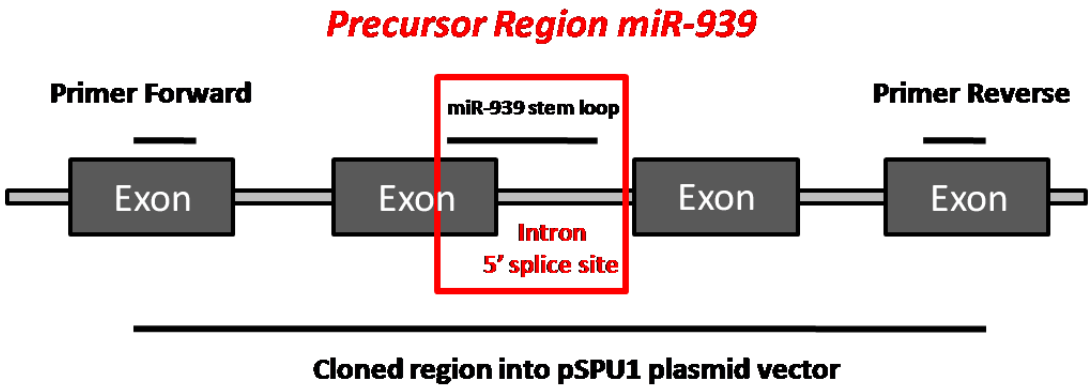


Figure 3.2.6.1.2b Scheme of the precursor region of miR-939-5p. The precursor region was cloned inside miR-939-5p over-expressing plasmid, under the control of U1 promoter and U1 terminator. Black bars represent the position of the specific primers used for the cloning

procedure and for the RT-PCR reaction. Red square indicates the precursor region of miR-939-5p overlapping the 5'splice site.

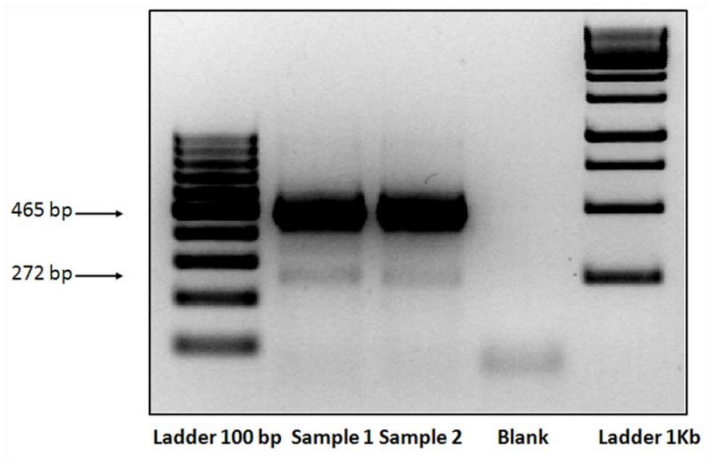


Figure 3.2.6.1.2c RT-PCR reaction of miR-939-5p precursor region. Figure of the 2% agarose gel where samples derived from the amplification of the precursor region of miR-939-5p were loaded. RT-PCR reactions performed with the RNA extracted from cells that were transfected with miR-939-5p over-expressing plasmid. Sample 1 and 2 are showing the amplification of the unspliced region cloned inside the over-expressing plasmid for miR-939-5p (465 bp band) and the spliced product of the same region (272 bp band). Blank represents the specific control of the RT-PCR reaction that does not contain the cDNA sample.

After the validation of the miRNAs over-expression, luciferase analyses were performed at 24h and 48h from the transfection day. In particular after 24h, there was a down-regulation correlated with every miRNA overexpressed and a significant reduction was observed after transfection with miR-615-5p, miR-939-5p and miR-659-3p compared to miR-181a (**Figure 3.2.6.1a**). At 48h there was an enhanced luciferase down-regulation for miR-615-5p, miR-939-5p and miR-608, compared to the negative control. However there was a slightly increase on the results variability and only miR-659-3p showed a significant reduction of luciferase activity. (**Figure 3.2.6.1b**).

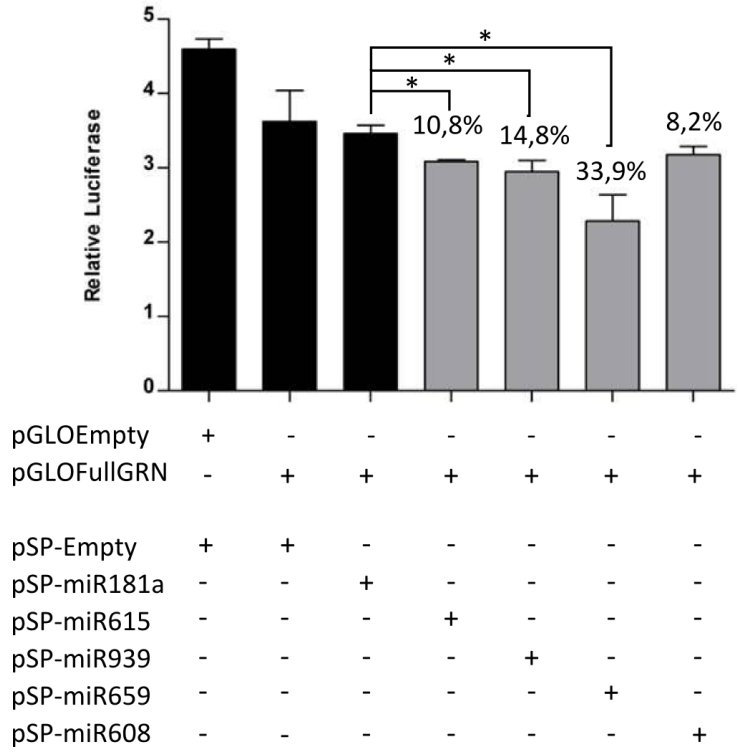


Figure 3.2.6.1a Luciferase assay at 24h, cotrasfection of pmiRGLOFullGRN3'UTR and putative miRNAs. Relative luciferase activity was measured in HeLa cells transiently co-transfected with pmiRGLOFullGRN3'UTR and selected miRNAs (miR-181a, miR-615-5p, miR-939-5p, miR-659-3p and miR-608) over-expressing plasmids. Firefly luciferase activity was normalized with *Renilla* luciferase activity. Percentage of luciferase reduction, reported above the graph bars, are related to negative control, miR-181a. Mean ± SEM of three biological replicates is shown in the figure (*p<0,05).

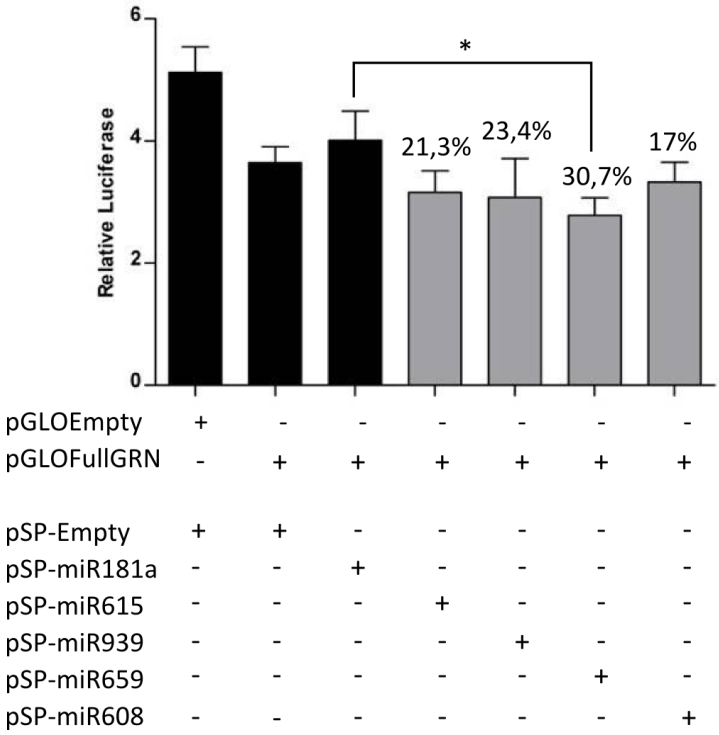
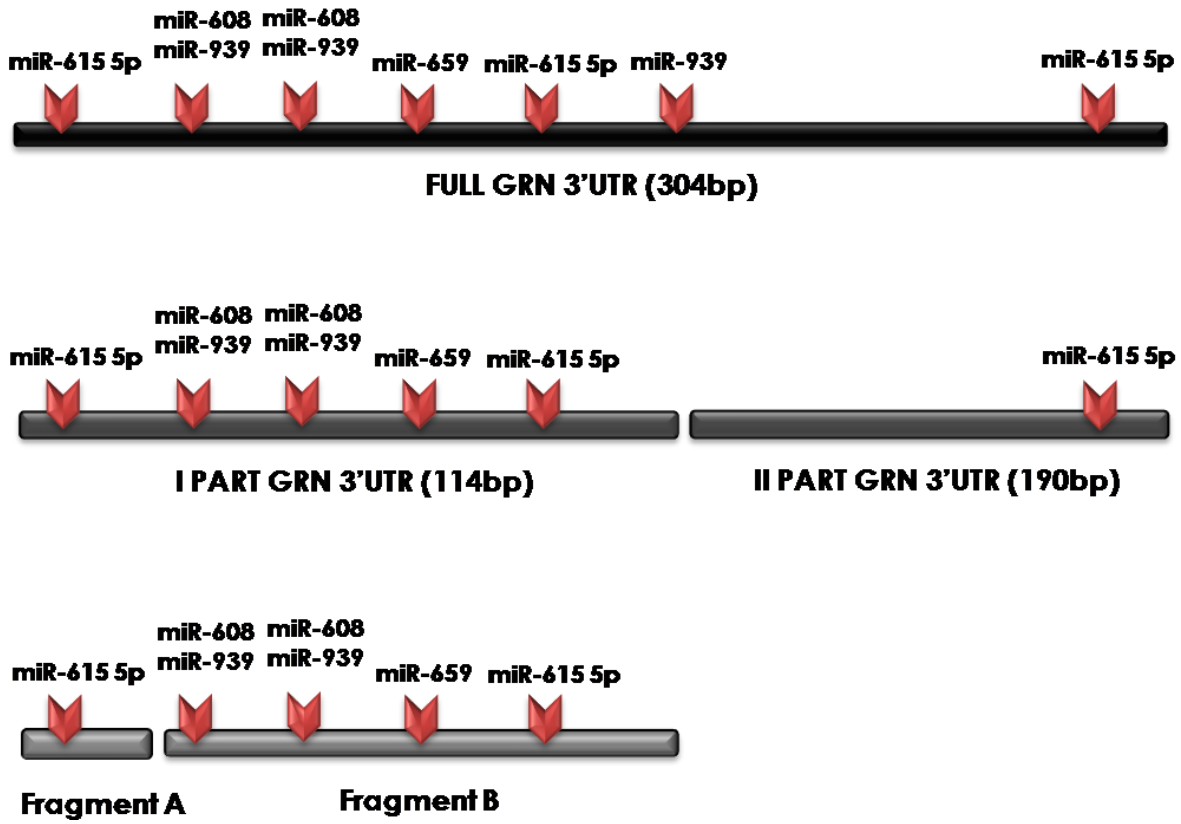


Figure 3.2.6.1b Luciferase assay at 48h, cotrasfection of pmiRGLOFullGRN3'UTR and putative miRNAs. Relative luciferase activity was measured in HeLa cells transiently co-transfected with pmiRGLOFullGRN3'UTR and selected miRNAs (miR-181a, miR-615-5p, miR-939-5p, miR-659-3p and miR-608) over-expressing plasmids. Firefly luciferase activity was normalized with *Renilla* luciferase activity. Percentage of luciferase reduction, reported above the graph bars, are related to negative control, miR-181a. Mean ± SEM of three biological replicates is shown in the figure(* $p < 0,05$).

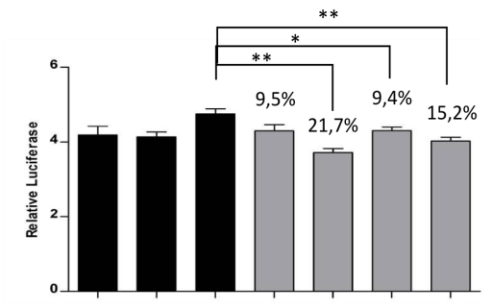
3.2.6.2 Luciferase Assays using reporter vectors containing different regions of GRN 3'UTR in HeLa cell line

In order to further investigate the different miRNAs contribution on the GRN regulation and to analyze the role of the different miRNAs binding sites, the GRN 3'UTR was divided in different parts, as represented in the **Scheme 3.2.6.2**. Luciferase assay analysis was performed at 24h and 48h after transfection. The **Figure 3.2.6.2. (A and C)** show a good level of luciferase reduction for all the selected miRNAs that have specific binding sites on the IPart of GRN 3'UTR (**A**) and on the fragment B of GRN 3'UTR (**C**). The reduction related to the IPart of GRN 3'UTR was significant at 24h and 48h. Whereas using the fragment B, the significant reduction was observed only at 48h, with a small and not significant down-regulation at 24h with all the selected miRNAs. Since these constructs are produced with different regions of the GRN 3'UTR, the secondary structure of these transcripts could play a role on the dynamic of miRNAs binding process, exposing or blocking specific sequences important for the miRNAs targeting. The IIPart of GRN 3'UTR contains only one putative binding site for miR-615-5p. Indeed at 24h and 48h there was a significant luciferase down-regulation only with miR-615-5p, while all the other miRNAs were not able to bind this specific region (**Figure 3.2.6.2. B**). In addition, the fragment A of GRN 3'UTR that contains only one binding site for miR-615-5p, showed a slightly luciferase reduction only at 24h, indicating a weak contribution for this miR-615-5p binding site on the regulation of this GRN 3'UTR region (**Figure 3.2.6.2. C**).



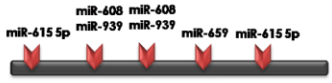
Scheme 3.2.6.2. GRN 3'UTR and different miRNAs binding sites. Schematic representation of the full length GRN 3'UTR with the specific binding sites of different miRNAs and the regions in which GRN 3'UTR was divided for the luciferase assays analysis.

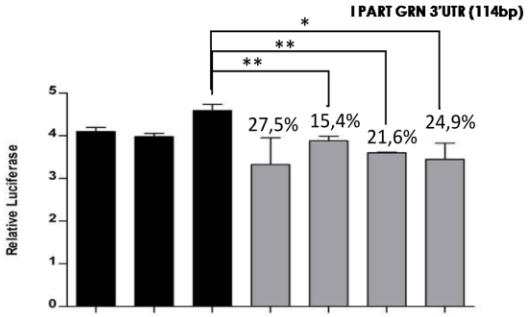
A I Part GRN 3'UTR Luciferase at 24h



pGLOEmpty	+	-	-	-	-	-	-
pGLOIPartGRN	-	+	+	+	+	+	+
pSP-Empty	+	+	-	-	-	-	-
pSP-miR181a	-	-	+	-	-	-	-
pSP-miR615	-	-	-	+	-	-	-
pSP-miR939	-	-	-	-	+	-	-
pSP-miR659	-	-	-	-	-	+	-
pSP-miR608	-	-	-	-	-	-	+

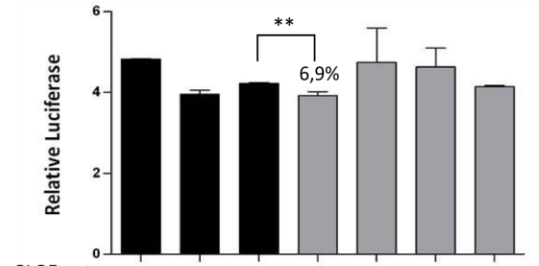
I Part GRN 3'UTR Luciferase at 48h





pGLOEmpty	+	-	-	-	-	-	-
pGLOIPartGRN	-	+	+	+	+	+	+
pSP-Empty	+	+	-	-	-	-	-
pSP-miR181a	-	-	+	-	-	-	-
pSP-miR615	-	-	-	+	-	-	-
pSP-miR939	-	-	-	-	+	-	-
pSP-miR659	-	-	-	-	-	+	-
pSP-miR608	-	-	-	-	-	-	+

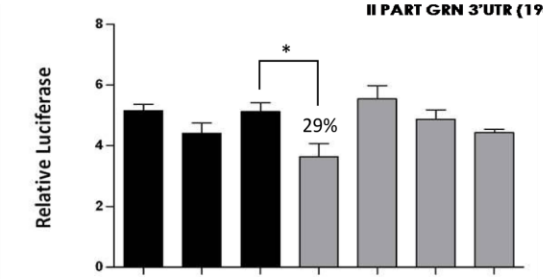
B II Part GRN 3'UTR Luciferase at 24h



pGLOEmpty	+	-	-	-	-	-	-
pGLOIIPartGRN	-	+	+	+	+	+	+
pSP-Empty	+	+	-	-	-	-	-
pSP-miR181a	-	-	+	-	-	-	-
pSP-miR615	-	-	-	+	-	-	-
pSP-miR939	-	-	-	-	+	-	-
pSP-miR659	-	-	-	-	-	+	-
pSP-miR608	-	-	-	-	-	-	+

Fragment A

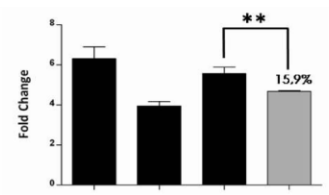
II Part GRN 3'UTR Luciferase at 48h



pGLOEmpty	+	-	-	-	-	-	-
pGLOIIPartGRN	-	+	+	+	+	+	+
pSP-Empty	+	+	-	-	-	-	-
pSP-miR181a	-	-	+	-	-	-	-
pSP-miR615	-	-	-	+	-	-	-
pSP-miR939	-	-	-	-	+	-	-
pSP-miR659	-	-	-	-	-	+	-
pSP-miR608	-	-	-	-	-	-	+

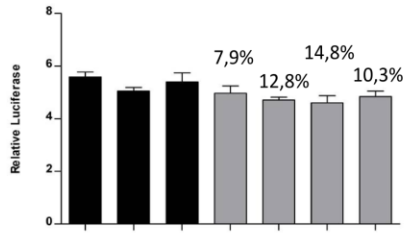
Fragment B

C Fragment A GRN 3'UTR Luciferase at 24h



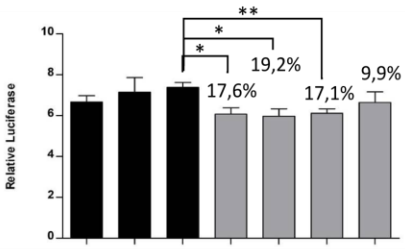
pGLOEmpty	+	-	-	-
pGlO-Frag.A	-	+	+	+
pSP-Empty	+	+	-	-
pSP-miR181a	-	-	+	-
pSP-miR615	-	-	-	+

Fragment B GRN 3'UTR Luciferase at 24h



pGLOEmpty	+	-	-	-	-	-	-
pGLOFrag.B	-	+	+	+	+	+	+
pSP-Empty	+	+	-	-	-	-	-
pSP-miR181a	-	-	+	-	-	-	-
pSP-miR615	-	-	-	+	-	-	-
pSP-miR939	-	-	-	-	+	-	-
pSP-miR659	-	-	-	-	-	+	-
pSP-miR608	-	-	-	-	-	-	+

Fragment B GRN 3'UTR Luciferase at 48h



	+	-	-	-	-	-	-
	-	+	+	+	+	+	+
	+	+	-	-	-	-	-
	-	-	+	-	-	-	-
	-	-	-	+	-	-	-
	-	-	-	-	+	-	-
	-	-	-	-	-	+	-
	-	-	-	-	-	-	+

Figure 3.2.6.2. Luciferase assays of different regions of the GRN 3'UTR. A) Luciferase analysis performed at 24h and 48h, using pmiRGLO vector containing the IPart of GRN 3'UTR and all the over-expressing plasmids for the putative miRNAs. **B)** Luciferase analyzed at 24h and 48h, using pmiRGLO vector containing the IIPart of the GRN 3'UTR and all the over-expressing plasmids for the putative miRNAs. Percentage of luciferase reduction, reported above the graph bars, are relative to miR-181a. Data derived from three biological replicate experiments (Mean ±SEM). **C)** Luciferase analysis performed at 24h, using pmiRGLO vector containing the fragment A of the GRN 3'UTR and the over-expressing plasmid for miR-615-5p-5p. Data representative of two biological replicate experiments (Mean ±SEM). Luciferase assays performed at 24h and 48h, using pmiRGLO vector containing the fragment B of the GRN 3'UTR and the over-expressing plasmid for all the putative miRNAs.Data derived from three biological replicate experiments (Mean ±SEM). (* P<0,05, ** P<0,001).

3.2.7 Effect of Selected miRNAs Over-expression on Progranulin level in KELLY cell line

The level of progranulin protein was measured using western blot analysis after miRNAs over-expression. KELLY cell line was transfected with the over-expressing plasmid for putative miRNAs entire panel and the backbone empty plasmid, used for miRNA cloning, as negative control. The efficiency of miRNAs over-expression was analyzed with TaqMan qRT-PCR, using RNA extracted from parallel samples, that underwent the same transfection experiments. In **Figure 3.2.7a** the basal endogenous level of expression of selected miRNAs (light grey) is compared to the level of miRNAs over-expressed plasmids (dark grey). Moreover the ΔΔCt values were calculated using as normalizers the miRNA levels measured after transfection of backbone empty plasmid, utilized for miRNA cloning. These fold changes are reported in the **Figure 3.2.7b.**

Proteins were extracted and used for western blot analysis as described in the Material and Methods section. HPRT protein was detected with specific antibody and used to normalize progranulin expression in all the analyzed conditions. Compared to the endogenous level, the entire panel of over-expressed miRNAs produced a decrease of progranulin protein. Percentage of progranulin expression is represented in **Figure 3.2.7c.** In particular the transfection of miR-615-5p leaded to 71,7% of progranulin expression, miR-608 leaded to 81,6% of progranulin expression. Stronger effect of protein down-regulation was obtained with the over-expression of miR-659-3p and miR-939-5p, with respectively 64,9% and 64,7% of progranulin expression**.**

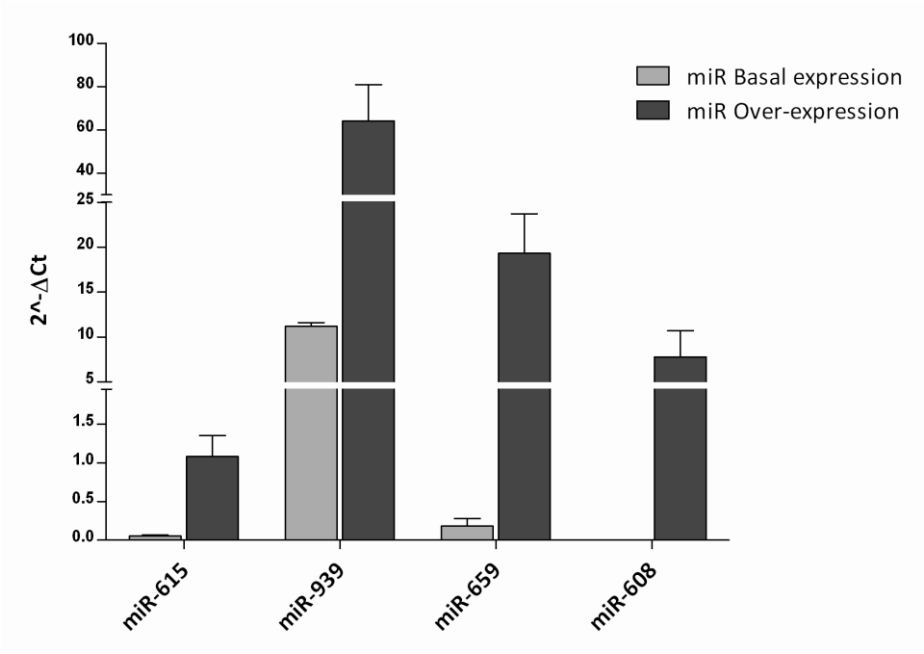


Figure 3.2.7a Basal and Over-expression levels of selected miRNAs in KELLY cell line. TaqMan real time PCR was performed on the RNA extracted after 48h from cells transfected with over-expressing miRNA plasmids. C_T levels are normalized against RNU6 to calculate the fold change with the $2^{-\Delta Ct}$ method. Data are obtained from biological duplicates and technical triplicates (Mean ±SD).

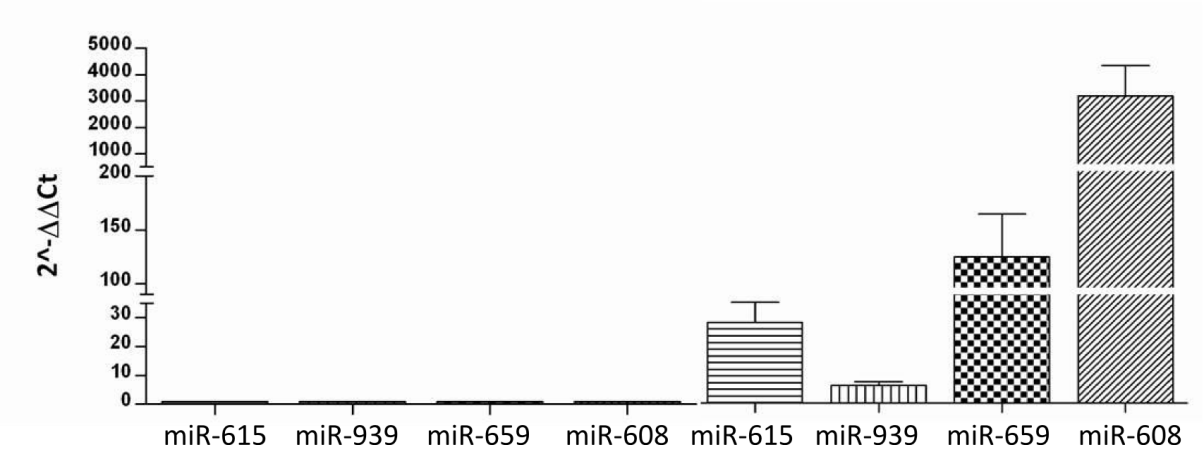


Figure 3.2.7b Over-expression levels of selected miRNAs in KELLY cell line. Representation of the $2^{-\Delta\Delta Ct}$ values obtained through the comparison between the over-expression of miRNAs and the endogenous basal level of expression in KELLY cell line. Data represent the summary of biological duplicates and technical triplicates (Mean ±SD).

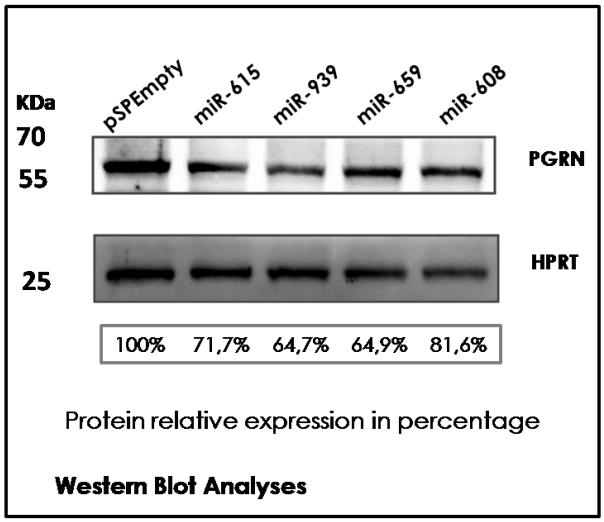


Figure 3.2.7c Effect of miRNAs over-expression on progranulin. The over-expression of the putative miRNAs downregulated the endogenous progranulin protein expression represented by the sample transfected with pSP-Empty vector. Sample transfected with pSP-Empty vector were used as control to calculate the percentage of progranulin expression, reported below the images. Data normalized with HPRT protein expression. Representative western blot of an experiment repeated three times.

3.2.8 Effect of miR-939-5p Inhibition on Progranulin Expression in HeLa cell line

Quantification of miRNAs basal expression showed that miR-939-5p is expressed in a good level in HeLa cells at physiological condition. For this reason a specific miR-939-5p inhibitor, based on locked nucleic acid (LNA) technology was used to block the basal expression of miR-939-5p and subsequently analyzed progranulin expression using western blot. After 48h from the transfection of the miR-939-5p inhibitor and the specific control, proteins were extracted and used for western blot analysis. GAPDH protein was detected with specific antibody and used to normalize progranulin expression. Compared to the control level, progranulin expression was found increased with the transfection of miR-939-5p inhibitor. Percentage of progranulin expression is represented in **Figure 3.2.8** . Specifically progranulin expression increased up to 124% compared to the basal level. This result suggests a possible regulation played by miR-939-5p on progranulin expression.

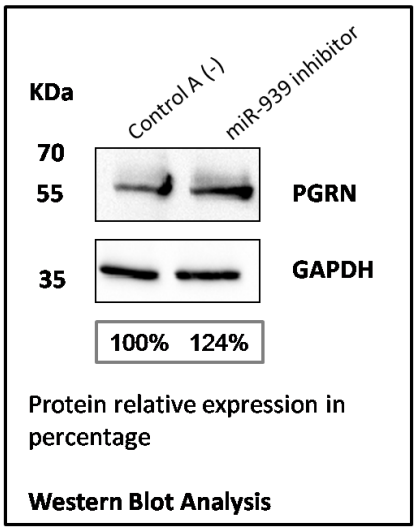


Figure 3.2.8 Effect of miR-939-5p inhibitor on progranulin expression. Inhibition of miR-939-5p caused an up-regulation of progranulin expression, compared to control. Sample transfected with a specific control vector was used to calculate the percentage of progranulin expression, reported below the images. Data normalized with GAPDH protein expression.*

3.2.9 Effect of Selected miRNAs Over-expression on GRN mRNA level in KELLY cell line

In order to determine if miRNAs over-expression can have an effect on the GRN mRNA levels, KELLY cells were transfected with over-expressing plasmid for all the putative miRNAs of interest and, as a negative control, the plasmid that does not contain any miRNA precursor region. RNA was extracted after 48h from the transfection day and GRN mRNA levels were measured by quantitative RT-PCR and normalized with two housekeeping genes: the hypoxanthine phosphoribosyltransferase I (HPRTI) and the succinate dehydrogenase complex subunit A (SDHA). The fold changes were calculated with the $2^{-\Delta\Delta Ct}$ method, comparing the levels of GRN mRNA present in the samples where specific miRNAs were over-expressed with the levels of GRN mRNA measured in the samples transfected with the negative control, the empty vector (**Figure 3.2.8**). Only samples transfected with the over-expressing plasmid for miR-615-5p showed a significant down-regulation of GRN mRNA level, whereas the cells transfected with the other putative miRNAs had a small and not significant down-regulation of GRN mRNA level compared to negative control.

*This work was performed in collaboration with Dr.Margherita Grasso, at the laboratory of RNA Biology and Biotechnology, CIBIO

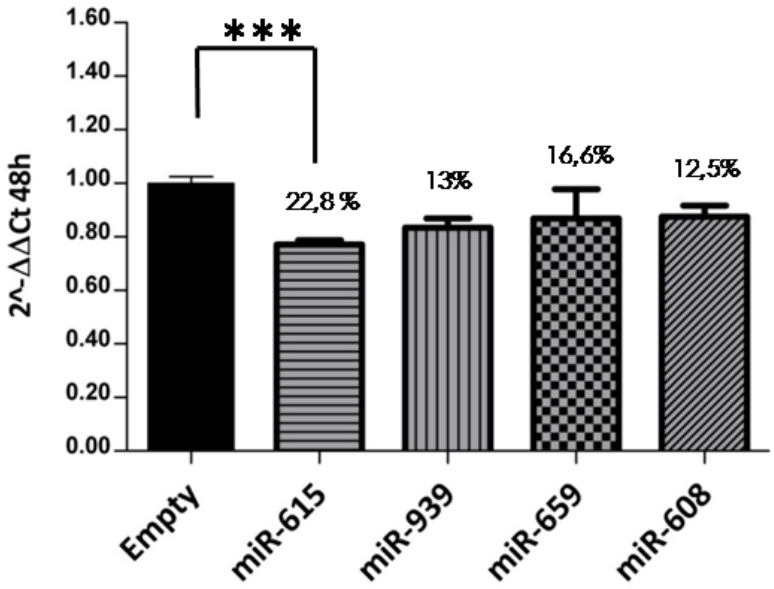


Figure 3.2.9 Effect of miRNAs over-expression on the GRN mRNA level. KELLY cells were transfected with the putative miRNAs and the empty control. RNA was extracted after 48h and analyzed through real time PCR for the detection of GRN mRNA and two specific normalizators, SDHA and HPRTI mRNAs. Representation of the fold changes calculated with the $2^{-\Delta\Delta Ct}$ method. Data derived from biological duplicate experiments (Mean ±SEM).(*** P<0,0001).

3.2.10 Effect of miRNAs Over-expression on GRN mRNA in polysomal and sub-polysomal compartments in KELLY cell line

The over-expression of the miRNAs of interest seems to reduce progranulin protein level, but not strongly alter and degrade the GRN mRNA level. Therefore, to verify whether the selected miRNAs regulate the level of progranulin by affecting the translation efficiency of GRN mRNA, KELLY cell line was transfected with the miRNAs over-expressing vectors and the empty vector as control. 48h after transfection the mRNA-ribosomal complexes were separated through sucrose gradient centrifugation in polysomal and sub-polysomal fractions containing the effectively translated transcripts and the untranslated mRNAs respectively. For each transfection condition, RNA was extracted and a Real Time PCR was performed in order to quantify the GRN mRNA amount in both polysomal and sub-polysomal compartments. The expression of HPRTI mRNA was used as normalizator to calculate the $2^{-\Delta Ct}$ values and results are represented in **Figure 3.2.10b**. The relative distributions of GRN mRNA in the two compartments were indicated as percentages calculated on the sum of the transcript extracted from the two fractions, for each treatment and control. The miRNAs over-expression led to an increase of GRN mRNA presence in the sub-polysomal parts compared to the control. Indeed the experiments showed a trend toward a shift of GRN mRNA from the polysomal to the sub-polysomal compartment after the transfection with miRNAs compared to the empty control. These results indicate that the selected miRNAs could regulate the level of progranulin by affecting the translation efficiency of GRN mRNA (**Figure 3.2.10b**).

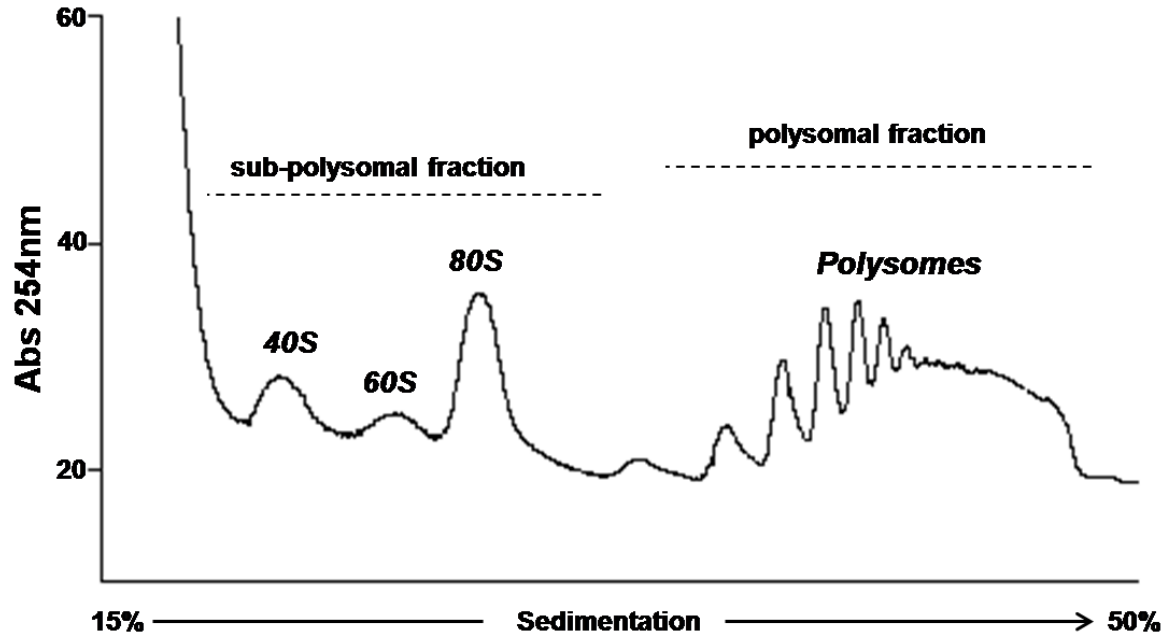


Figure 3.2.10a Polysomal Profile. Representative polysome profile obtained from KELLY cell line. The figure showed 40S and 60S- free small and large ribosomal subunits, 80S-monosomes and the sub-polysomal and polysomal fractions.*

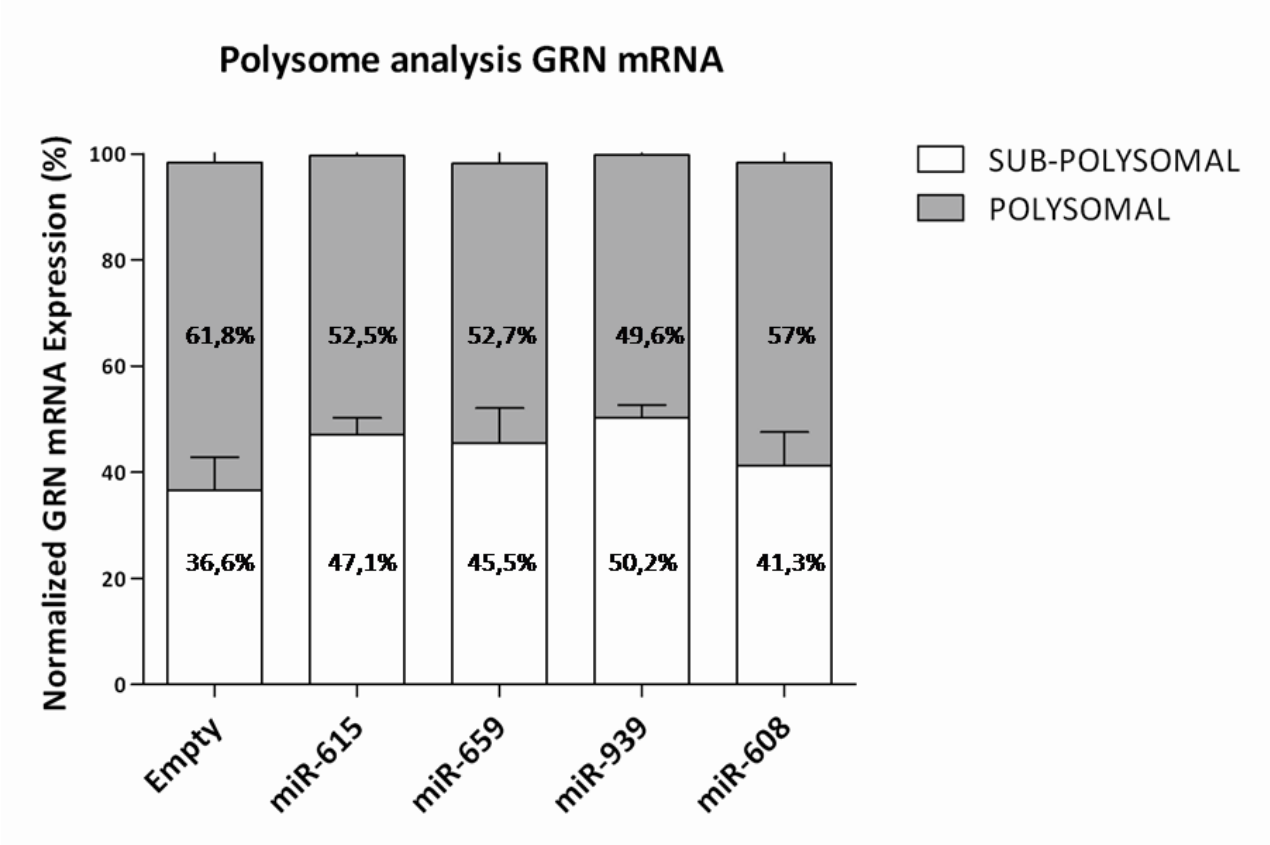


Figure 3.2.10b Effect of miRNAs over-expression on GRN mRNA in polysomal and sub-polysomal compartments. KELLY cell line was transfected with the over-expressing plasmid for all the selected miRNAs and the empty control. After 48h the mRNA-ribosomal complexes were separated through sucrose gradient centrifugation and RNA was extracted from the sub-polysomal and polysomal parts. Real Time PCR was performed for the quantification of GRN mRNA in the sub-polysomal and polysomal fractions. GRN mRNA expression was normalized against the housekeeping HPRTI transcript. Percentages of GRN transcript measured in the two fractions are calculated on the sum of $2^{-\Delta Ct}$ values of GRN mRNA from polysomal and sub-polysomal compartments. Data derived from three biological experiments. (Mean ±SEM).*

*This work was done in collaboration with Dr. Paola Zuccotti from laboratory of Translational Genomics in CIBIO.

CHAPTER FOUR: PROGRANULIN AND HYPOXIA*

4.1 Introduction

In CNS, normoxia constitutes the situation in which the level of O_2 is considerably lower than the level of O_2 present in the systemic circulation (Peers *et al.* 2009). Indeed, with an arterial O_2 level of 90 mm Hg, it is reported that the cerebral cortex is functional at a pO_2 below 30 mm Hg, with 50% functioning below 10 mm Hg (Sick *et al*. 1982; LaManna *et al*. 2007). However, the brain can undergo through relative hypoxia conditions due to pathological situations that could derive from different respiratory or cardiovascular diseases (Peers *et al.* 2007). Neurons are vulnerable to oxygen changes and the brain shows neural damage after few minutes of oxygen deprivation (Bazan *et al*. 2002). Increasing evidences indicated association between neurodegeneration and hypoxia. Indeed dementia represents a risk factor for the survival of stroke patients (Desmond *et al*. 2002a), while stroke patients are more prone to develop AD during the following years (Desmond *et al.* 2002b). It was observed that *in vivo* hypoxia caused an increase levels of amyloid precursor protein (APP), that is the substrate for the amyloid β peptide (Aβ) aggregates classically present during Alzheimer's disease pathology (Skoog *et al.* 2006). Moreover Aβ peptides induce the formation of reactive oxygen species (ROS) (Neve *et al*. 1998; Daniels *et al*. 2001). Increased ROS expression is also associated with different neurodegenerative diseases such as Parkinson's disease, or other disorders such as arthritis, stroke and atherosclerosis (Aliev *et al*. 2002). Furthermore infants that died from hypoxia showed neuronal degeneration in the hippocampus region with the presence of AD-type astrocytic changes (Nakamura *et al*. 1986). Therefore hypoxia seems not only an important factor for the generation and the progression of AD, but could be responsible for the pathogenesis of FTD (Martin *et al*. 2001). This hypothesis is supported by the facts that hypoxia in an acidic environment and ischemia caused astrocytes degeneration in vitro (Martin *et al*. 2001). In addition in FTD cases it was shown an inverse correlation between astrodegeneration and cerebral blood flow, indicating that mild ischemia could possibly contribute to the progression of FTD (Martin *et al.* 2001). In this scenario progranulin was reported as a stress response factor under hypoxia and acidosis conditions. A work demonstrated that

fibroblasts cultures exposed to hypoxia and acidosis showed an increased progranulin expression (Guerra *et al*. 2007). An additional study reported that hypoxic injury could significantly affect progranulin expression. HT22 mouse hippocampal cell line under hypoxic condition showed an increase of progranulin expression within a shorter period with a decrease of progranulin levels observed after 6 hours (Luo *et al*. 2014). Levels of GRN mRNA and progranulin protein expression were found significantly elevated following chronic hypoxia in neuroblastoma cell lines (Piscopo *et al*. 2010). This progranulin up-regulation seems to be important for the rescue from cell death induced by oxidative insults or glutamate-induced excitotoxicity (Xu *et al*. 2011). The chronic exposure of neurons to these neurotoxic mechanisms has been suggested as important factor contributing to neurodegeneration diseases with the progressive loss of cortical neurons (Fatokun *et al*. 2008; Lau *et al.* 2010). Several studies demonstrated the neuroprotective role of progranulin (Xu *et al.* 2011; Martens *et al*. 2012; Zhou *et al.* 2015) that can stimulate the activation of the MEK/ERK/p90RSK and PI3K/Akt cell survival pathways after toxic insults (Xu *et al.* 2011). The absence of the neuroprotective activities observed in progranulin deficiency mice predisposes to an hyperactivation of microglia that contributes to increased neurons loss in response to oxidative injury (Martens *et al*. 2012). Indeed FTD patients that have a reduced expression of progranulin, showed microglia in an activate state (Ahmed *et al.* 2007). Therefore in condition of a reduced extracellular progranulin, like in a pathological FTD state, the neuroprotective signaling, induced by increased progranulin expression, will be less effective or absent with apoptotic or necrotic death. Even though neuroprotective function of progranulin was found more potent against the apoptotic death (Xu *et al*. 2011).

It is reported that cells in low-oxygen conditions induce changes in a specific family of miRNAs, called hypoxamirs (Nallamshetty *et al.* 2013). An important factor that can mediate these modifications is the hypoxia inducible factor (HIF) (Semenza, 2012). HIF is an heterodimeric transcription factor, that is activated under hypoxic conditions and recruited to bind consensus hypoxia responsive elements (HREs) in the promoters of different genes (Nallamshetty *et al.* 2013), including promoter for different miRNA genes (Kulshreshtha *et al*. 2007). Some hypoxamirs promote the activity of HIF (Loscalzo, 2010), or are direct targets of HIF during hypoxia, such as miR-210, that is induced by hypoxia in a HIF-dependent way and stabilizes HIF expression (Kelly *et al*. 2011). Other hypoxamirs induce adaptive actions to hypoxia in a way that is synergistic with the role played by HIF (Crosby *et*

al. 2009). However during hypoxic stress, other different transcriptional factors could have important role, such as NF-KB, p53 (Cummins *et al*. 2005). For these reasons hypoxamir biogenesis could be induced through an HIF-dependent or HIF-independent way.

The present work intended to investigate the role played by miR-659-3p in the regulation of progranulin during oxidative stress. Previous analysis of our collaborators suggested that perinatal asphyxia causes a transient oxidative stress followed by an altered expression of different genes involved in antioxidant defenses, brain development and neuroprotection (Piscopo *et al.* 2008). As other reported, they observed that chronic hypoxia leads to an increased expression of GRN mRNA and progranulin protein in neuroblastoma cell lines (Piscopo *et al*. 2010). Therefore we wondered if the putative miRNAs implicated in the regulation of GRN 3'UTR could have a role in the upregulation of progranulin expression during hypoxia.

*****This work is part of a collaborative project with the Department of Cell Biology and Neuroscience, Istituto Superiore di Sanità in Rome. For the complete project refer to the attached manuscript "**Reduced miR-659-3p levels correlate with progranulin increase in hypoxic conditions: implications for frontotemporal dementia"** submitted to the journal "Molecular and Cellular Neuroscience".

4.2 Results

4.2.1 Functional assays to validate miR-659-3p binding on the GRN 3'UTR

In order to analyze the binding of miR-659-3p on GRN 3'UTR, HeLa cell line was co-transfected with reporter plasmid containing the full length GRN 3'UTR downstream the firefly luciferase gene (pmiRGLO-GRN3'UTR) and the over-expressing plasmid for miR-659-3p (pSiUx-miR659). The level of luciferase activity was compared to the level obtained with the transfection of an over-expressing vector for a non-targeting miRNA, representing the negative control (pSiUx-miR181a). The production of mature miRNAs derived from the over-expressing plasmids was quantified by TaqMan Real Time PCR at 24h and 48h, as reported in the **Figure 4.2.1a.** The levels of miR-659-3p, were increased of 315 times at 24h and of 123 times at 48h. Whereas the levels of miR-181a, that was already highly expressed in HeLa cell line at basal condition, were increased of 2,4 times at 24h and 2,4 times at 48h.

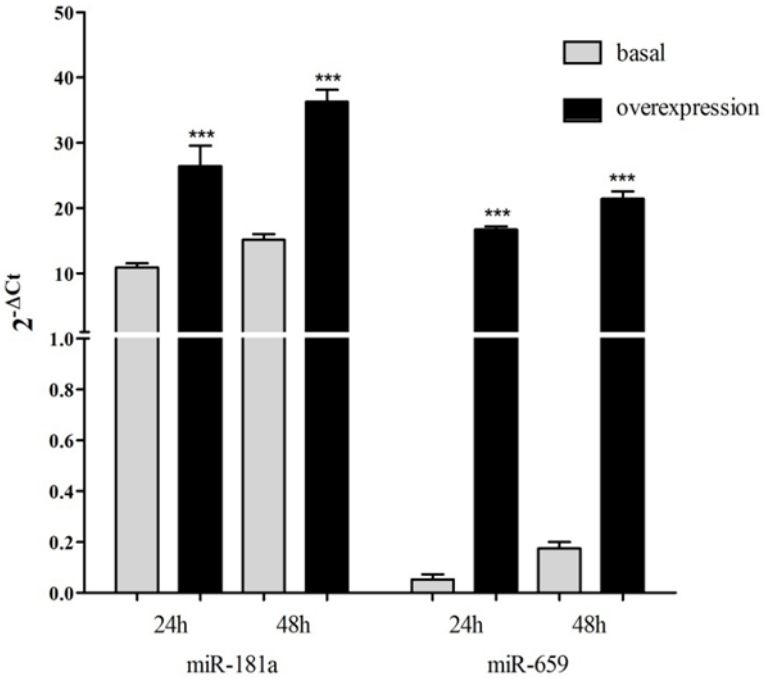


Figure 4.2.1a pSiUx-miR-659 and pSiUx-miR-181a over-expression at 24h and 48h. miRNAs expression levels measured by qRT-PCR after 24h and 48h from transfection. Mean ± SEM of two biological replicates is shown (N=6, ***$p < 0.001$ vs. miRNAs basal expression levels).

Co-transfection experiments with pmiRGLO-GRN'3'UTR and the over-expressing plasmids for miR-659-3p and miR-181a led to a significant level of luciferase down-regulation. In particular transfection of miR-659-3p caused a luciferase reduction of 33,9%, compared to miR-181a at 24h (**Figure 4.2.1b A**), whereas transfection with miR-659-3p at 48h produced a decreased luciferase activity of 30,7%, compared to the luciferase level measured with miR-181a transfection (**Figure 4.2.1b B**).

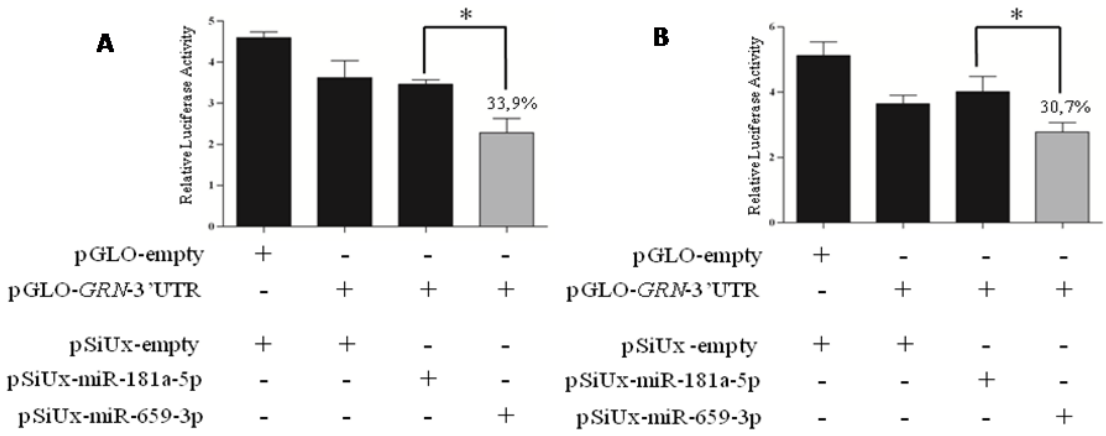


Figure 4.2.1b. Luciferase assays confirm the interaction between miR-659-3p and *GRN* 3'-UTR. *GRN* 3'-UTR was cloned downstream of the firefly luciferase gene in pmiRGLO vector and co-transfected with miR-659 and miR-181a-overexpressing plasmids in HeLa cells. Luciferase activity was assessed at 24h (**A**) and 48h (**B**) after transfection (* $p < 0.05$).

4.2.2 Effect of miR-659-3p over-expression on progranulin protein, analyzed with ELISA and western blot

The effect of miR-659-3p over-expression on the endogenous progranulin protein was investigated in HeLa cell line after 48h from the transfection. The levels of progranulin protein, measured after the transfection of miR-659-3p over-expressing plasmid, was compared to progranulin levels quantified after the transfection of the over-expressing plasmid for miR-181a, as represented in the **Figure 4.2.2**. This analysis was performed with two different techniques, ELISA (**Figure 4.2.2 A**) and western blot (**Figure 4.2.2 B**). In the ELISA assays miR-659-3p induced a progranulin reduction average of 25% compared to miR-181a (**Figure 4.2.2 A**). Whereas in the western blot analysis miR-659-3p led a progranulin

down-regulation of 15% compared to the negative control, miR-181a (**Figure 4.2.2 B**). These figures were obtained from four and three biological replicates, respectively.

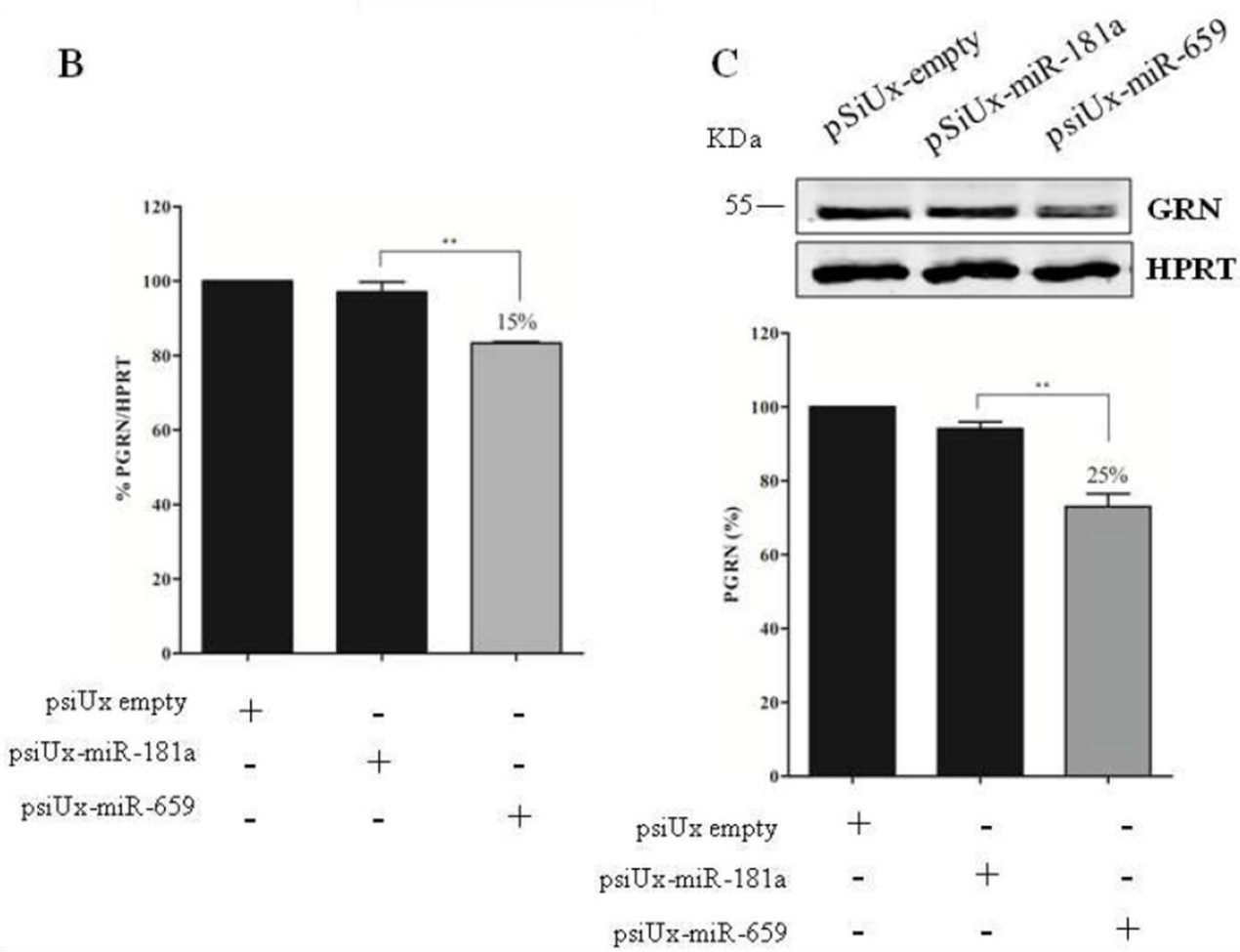


Figure 4.2.2. Effect of miR-659-3p over-expression on progranulin protein in HeLa cell line. ELISA assay (**A**) for progranulin quantification in protein extracts from HeLa cells (N=4; ** $p < 0.01$).Western blot analysis (**B**) for progranulin detection in protein extracts from HeLa cells (N=3; ** $p < 0.01$). Western Blot image is representative of three biological replicates. Expression of Western Blot was normalized on HPRT expression.

4.2.3 Validation of the physical interaction between miR-659-3p and GRN mRNA

In order to validate the effective interaction between miR-659-3p and GRN mRNA, miR-CATCH experiments were perfomed as described previously. The pull down assay was done on SK-N-BE cell line at physiological condition. The eluted samples were analyzed with Real Time PCR for the quantification of GRN mRNA present on capture samples compared to specific scrambled controls. GAPDH expression was used as normalizator. The **Figure 4.2.3 A**

represents the fold enrichment calculated with the $2^{-\Delta\Delta Ct}$ method. GRN mRNA was 23,73-fold enriched, compared to scrambled controls. The eluted samples were also analyzed with TaqMan real time for the quantification of miR-659-3p present on the capture samples compared to controls, as reported in the **Figure 4.2.3 B**. MiR-659-3p showed a 311,5-fold enrichment, compared to the quantity of miRNA present on scrambled control samples, calculated with $2^{-\Delta Ct}$ method.

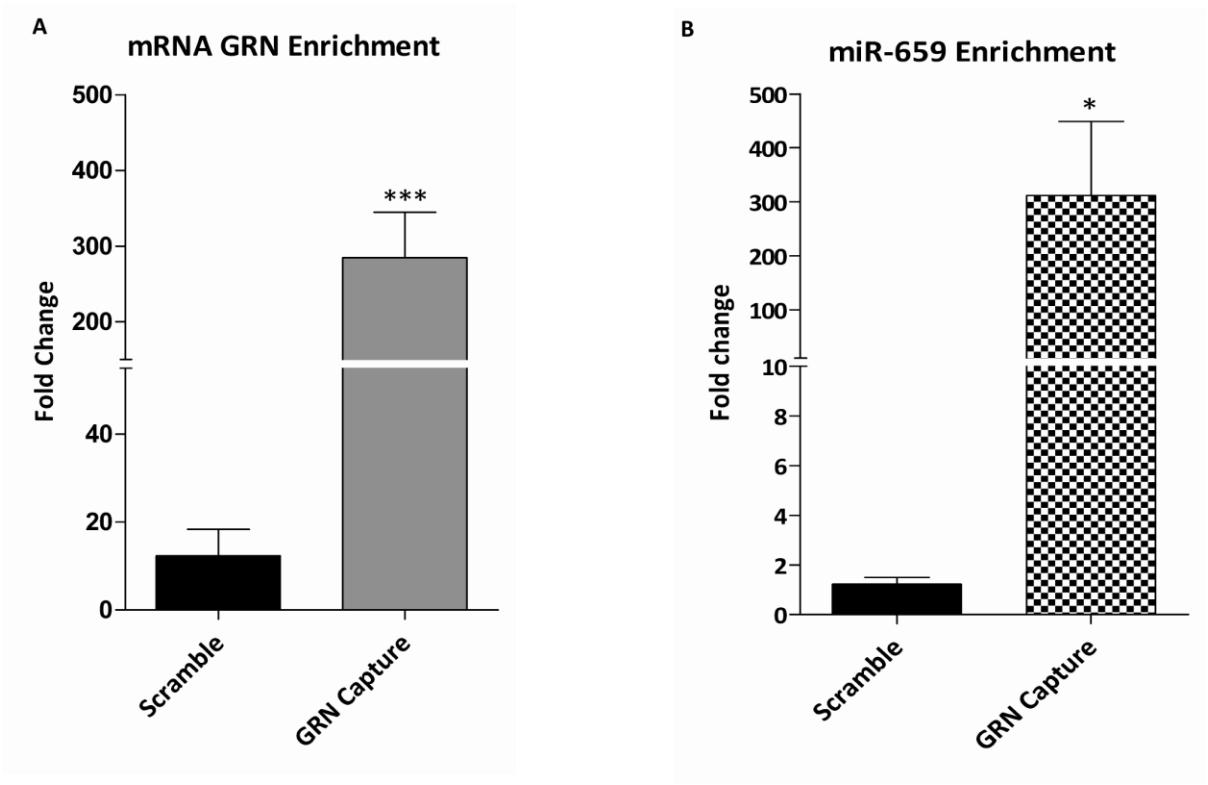


Figure 4.2.3. mRNA:miRNA isolation technique for *GRN* mRNA from SK-N-BE cell line. A capture anti-sense DNA oligonucleotide with a biotin modification at the 5' end was designed to pull-down *GRN* mRNA. qRT-PCR showed enrichment of *GRN* mRNA (**A**) and of miR-659-3p (**B)** in samples captured with *GRN* mRNA-specific oligonucleotide compared to a scrambled oligonucleotide used as negative control. mRNA expression was normalized with GAPDH expression and the fold enrichment was calculated with $2^{-\Delta\Delta Ct}$ method. miRNA fold enrichment was quantified with $2^{-\Delta Ct}$ method. Mean ± SEM of three biological replicates is shown (N=9, *$p < 0.05$; ***$p < 0.001$ vs. scrambled).

4.2.4 GRN 3'UTR sequencing in HeLa and SK-N-BE cell lines

GRN 3'UTR was characterized through sequencing in two different cell lines used: HeLa and SK-N-BE. Interestingly, the GRN 3'UTR sequence present in SK-N-BE cell line showed the presence of the SNP rs5848 found in FTLD patients by Rademakers and

colleagues (Rademakers et al. 2008). The common genetic variation (78 C>T) represents a genetic risk factor for FTD. In the study they reported a stronger binding of miR-659-3p in the presence of the T allele compared to the presence of the wild type C allele. In **Figure 4.2.4** are represented the chromatograms of the region containing the common genetic variant, for cell lines analyzed. SK-N-BE cell line showed an heterozygous presence of both alleles (TC) with a higher presence of the T allele (**Figure 4.2.4 B**). Whereas, GRN 3'UTR sequence derived from HeLa cell line showed the presence of wild type CC alleles (**Figure 4.2.4 A**).

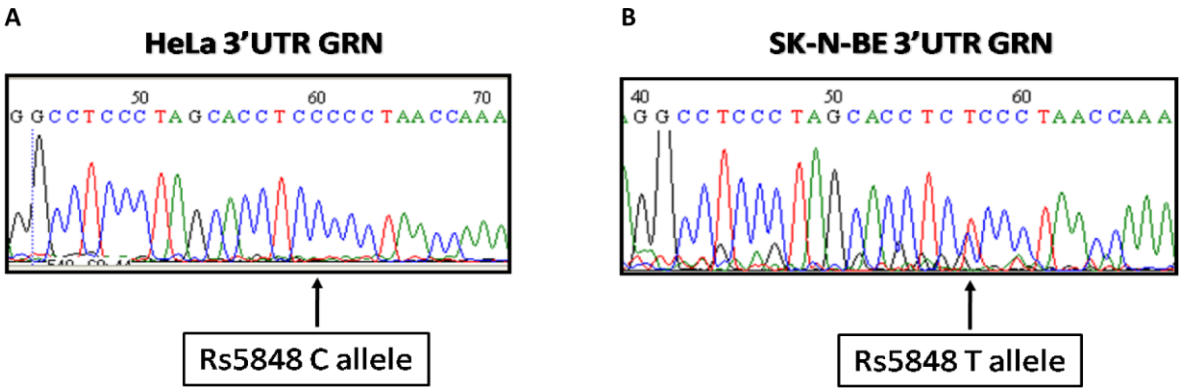


Figure 4.2.4 GRN 3'UTR sequencing in HeLa and SK-N-BE cell line. Representation of chromatograms of GRN 3'UTR sequencing performed in HeLa and SK-N-BE cell lines. The images report the region of GRN 3'UTR characterized by the common genetic variant rs5848 (78 C>T). HeLa cell line expresses wild type CC alleles (**A**), while SK-N-BE cell line shows heterozygous TC alleles, with the prevalence of the T allele (**B**).

4.2.5 Negative correlation between miR-659-3p expression and GRN mRNA levels in SK-N-BE cell line under hypoxic condition

SK-N-BE cell line was maintained in hypoxic condition for 24h. The glucose transporter GLUT1 was measured on the hypoxic SK-N-BE cell line, as marker of hypoxia. GLUT1 mRNA levels were found successfully up-regulated in the hypoxic samples compared to normoxic controls as reported in Figure 6 (Manuscript attached, Piscopo et al. 2015), demonstrating thus the correctly induced hypoxia condition. SK-N-BE hypoxic and normoxic samples were analyzed with TaqMan Real Time PCR for the quantification of miR-659-3p. Data were normalized with the expression of two small nucleolar RNAs, RNU44 and RNU48. These data were statistically investigated and correlated to the up-regulation of GRN mRNA expression observed on hypoxia samples compared to normoxic controls in Figure 6

(Manuscript attached, Piscopo et al. 2015). **Figure 4.2.5** represents the negative correlation between miR-659-3p and GRN mRNA expression found on SK-N-BE hypoxic samples compared to controls (Spearman's rank correlation coefficient = -0.96, p = 0.0005).

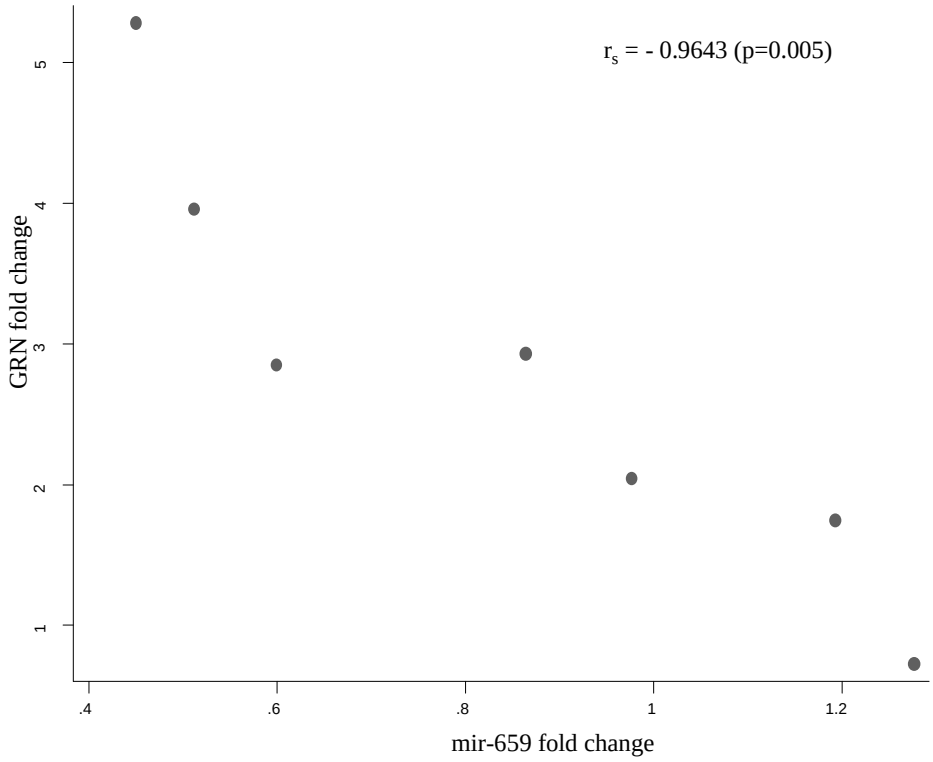


Figure 4.2.5 Negative correlation between GRN mRNA and miR-659-3p expression levels in SK-N-BE cell line.

4.2.6 Negative correlation between GRN mRNA and miR-659-3p in vivo

The correlation between GRN mRNA and miR-659-3p was analyzed in RNA samples derived from a rat model of global perinatal asphyxia. In a previous work it was demonstrated that 20 minutes of global asphyxia are necessary for the induction of significative brain oxidative stress (Calamandrei et al. 2004). The exposure conditions adopted in the present analysis were 0 and 20 minutes of asphyxia leading to a survival rate of 100% and 95%, respectively. qRT-PCR on GRN mRNA was performed in rat cortical samples at postnatal days (pnd) 1, 4 and 11. As reported in Figure 8 (Manuscript attached, Piscopo et al. 2015), 20 min of perinatal asphyxia was found to increase *GRN* mRNA levels compared to controls in all the analyzed pnd. The increase was significant at pnd1 and pnd4.

The same samples were analyzed with TaqMan Real Time PCR for the quantification of miR-659-3p. As represented in **Figure 4.2.6**, there was a decrease of miR-659-3p expression in rat cortical samples treated with perinatal asphyxia compared to controls, with a significative reduction at pnd4. Indeed pnd4 showed a strong up-regulation of GRN mRNA compared to non treated samples, indicating again a negative correlation between GRN transcript and miR-659-3p expression.

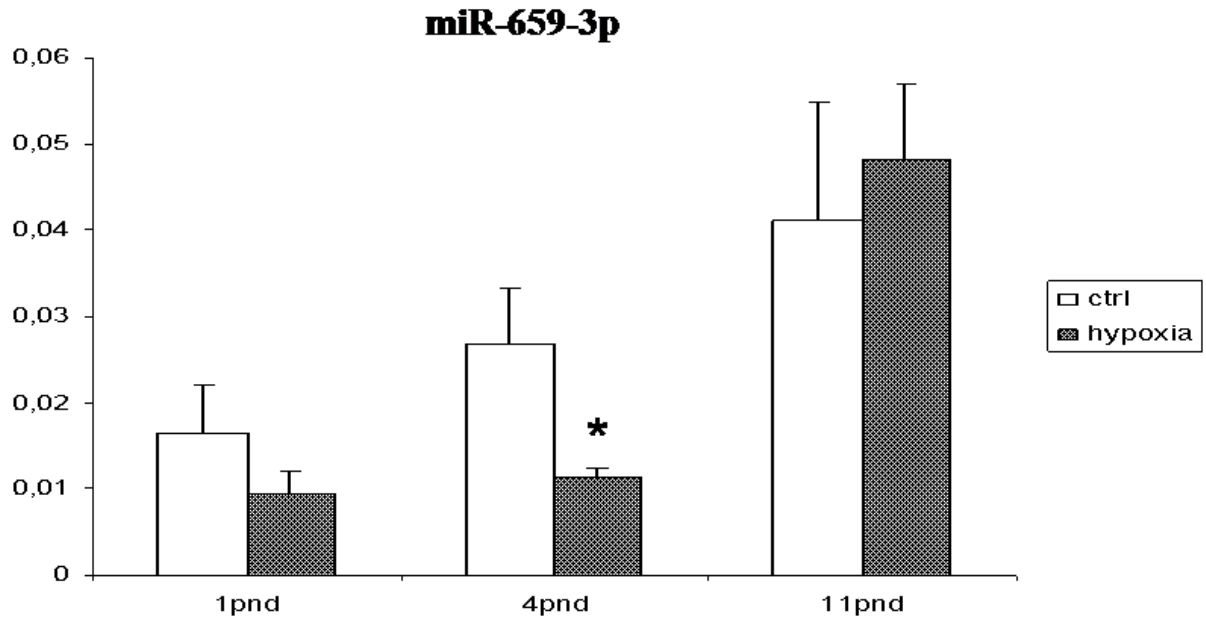


Figure 4.2.6 miR-659 down-regulation in cortex of asphyctic newborn rats. Levels of miR-659-3p measured by qRT-PCR at different time points in perinatal asphyctic newborn rats cortex and relative controls. Data, expressed as $2^{-\Delta Ct}$, are means±SEM. N=5

CHAPTER FIVE: IDENTIFICATION OF miRNAs INVOLVED IN THE POST-TRANSCRIPTIONAL REGULATION OF *MAPT* EXPRESSION

5.1 Introduction

The accumulation of tau protein is found and reported in a variety of different neurodegenerative disorders termed as tauopathies. These include the group of diseases collectively called as frontotemporal dementia (FTD), described previously (Wolfe, 2012). Tau is reported as the major component of the neurofibrillary tangles that accumulates in neurons in different type of tauopathies (Kosik *et al.* 1986, Wood and Mirra, 1986) and the presence of altered tau due to mutations, contributes to the process of neurodegeneration (Wolfe, 2009). In FTD, tau accumulations are usually found in the frontal and temporal lobes. The accumulations in these brain regions lead to socially inappropriate behavior and irrational choices (Lee *et al*. 2001). However, depending on the different type of FTD, tau aggregations can be present in other brain areas. Several experimental evidences suggested that a reduction of tau expression could represent a potential therapeutic strategy for the cure of tauopathies. Indeed knock out of tau in Alzheimer's Disease transgenic mouse models led to an improved learning and memory compared to mice with wild-type tau (Roberson *et al*. 2007; Ittner *et al*. 2010). Moreover, hippocampal neurons cultures derived from homozygous tau knockout mice did not show axonal transport defects (Vossel *et al.* 2010). Therefore, molecular approaches that lead to a reduction of tau expression, could represent possible therapeutic strategies for the cure of neurodegeneration caused by tau accumulations. A possible way to control gene expression is through the functions played by 3'UTR regions. Indeed 3'UTRs are involved in mRNA 3'end formation and polyadenylation at the pre-mRNA level, while they have a role in mRNA stability or degradation, nuclear export and translational efficiency at the mRNA level (Chen *et al*. 2006). The action carried out by miRNAs on this important regulatory region, can lead to mRNA degradation or the block of translation, causing strong effects on the cell physiology. So far the role of MAPT 3'UTR in the regulation of tau expression was quite understudied. Only two works identified that miR-34 family controls tau expression through the presence of binding sites located on the MAPT 3'UTR region (Wu *et al*. 2013; Dickinson *et al*. 2013;). In particular Dickinson and

collaborators found that tau expression could be regulated by miR-34a (Dickinson *et al*. 2013), whereas Wu and colleagues reported that miR-34c could down-regulate tau expression in gastric cancer cells (Wu *et al*. 2013). Even though miR-34a and miR-34c are encoded by different loci, they have the same seed, as members of the same family and they both bind on the same region on MAPT 3'UTR, regulating tau expression. Indeed LNA inhibition of endogenous miR-34 family led to an increase in tau expression (Dickinson *et al.* 2013). It is reported that 3'UTR regions are more prone to evolution, containing a higher density of short insertion/deletion length variants, single nucleotide polymorphisms (SNPs) and repeat expansions (Imanishi *et al*. 2004; Missirlis *et al*. 2005). Interestingly *MAPT* gene is part of an ancient inversion of an extended region that led to two haplotype H1 and H2. Even though the aminoacids sequence is unchanged, H1 and H2 are defined by a series of different SNPs and a deletion in intron 9 (Baker *et al.* 1999; Pittman *et al.* 2006). Since the structure of tau is not altered, any pathogenic effects associated with a particular haplotype could be investigated on the action of SNPs present on important regulatory regions, such as the 3'UTRs.

5.2 Results

5.2.1 MAPT mRNA as a direct target of putative microRNAs

As reported for GRN 3'UTR, three different bioinformatics softwares (PITA, Targetscan and Targetprofiler) were used to predict the putative miRNAs that have binding sites located on MAPT 3'UTR. In order to increase the specificity, the results derived from these bioinformatics tools were integrated to obtain a global list of putative miRNAs. Hundreds of miRNAs were predicted from each database, however the same putative miRNAs that were identified for the regulation of GRN 3'UTR, showed potential binding sites on MAPT 3'UTR. This finding suggest a possible common mechanism of regulation played by miRNAs on these two different 3'UTR regions. Since MAPT 3'UTR is 4160 bp in length, the selected miRNAs (miR-608, miR-939-5p, miR-615-5p and miR-659-3p) showed multiple binding sites with good score in terms of free binding energy, site accessibility and seed type. In particular,the putative binding sites found for our selected miRNAs are: 13 for miR-608, 16 for miR-939-5p, 7 for miR-615-5p-5p and 3 for miR-659-3p. In order to analyze the regulation played by these selected miRNAs, the MAPT 3'UTR was divided into eight different and partially overlapped fragments of 200 bp (MAPT 3'UTR I – VIII). The MAPT 3'UTR fragment V was chosen for the analysis of 4 miR-608 binding sites and 1 miR-659-3p binding site, as reported by the **Figure 5.2.1a.** To analyze the level of sequence conservation, MAPT 3'UTR sequences derived from different species (mouse, rat and human) were aligned with ClustalW program. The 3'UTR showed a relevant level of conserved regions, as reported in the **Figure 5.2.1b.**

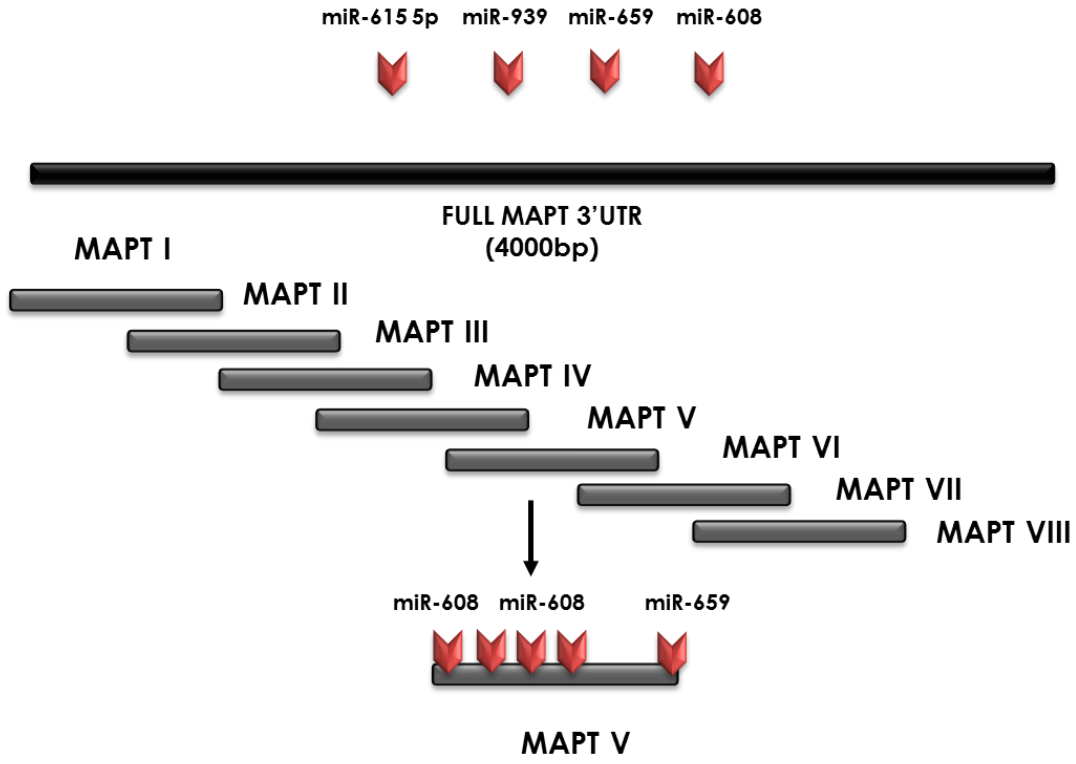
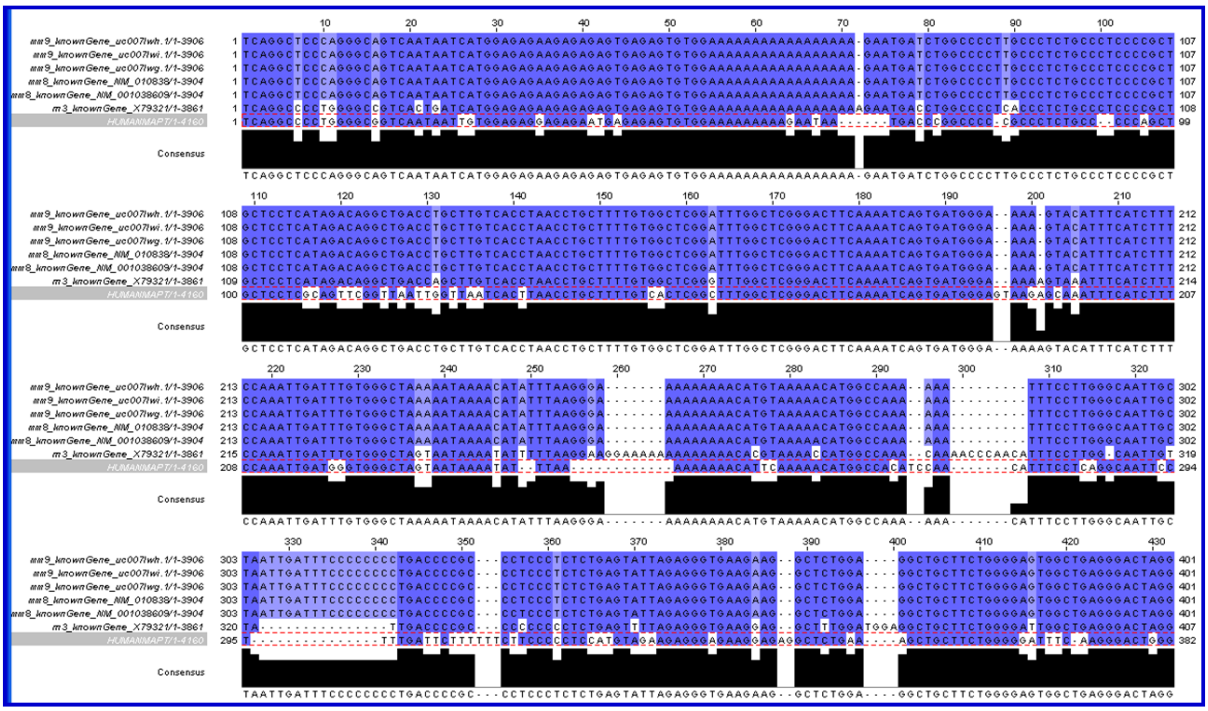
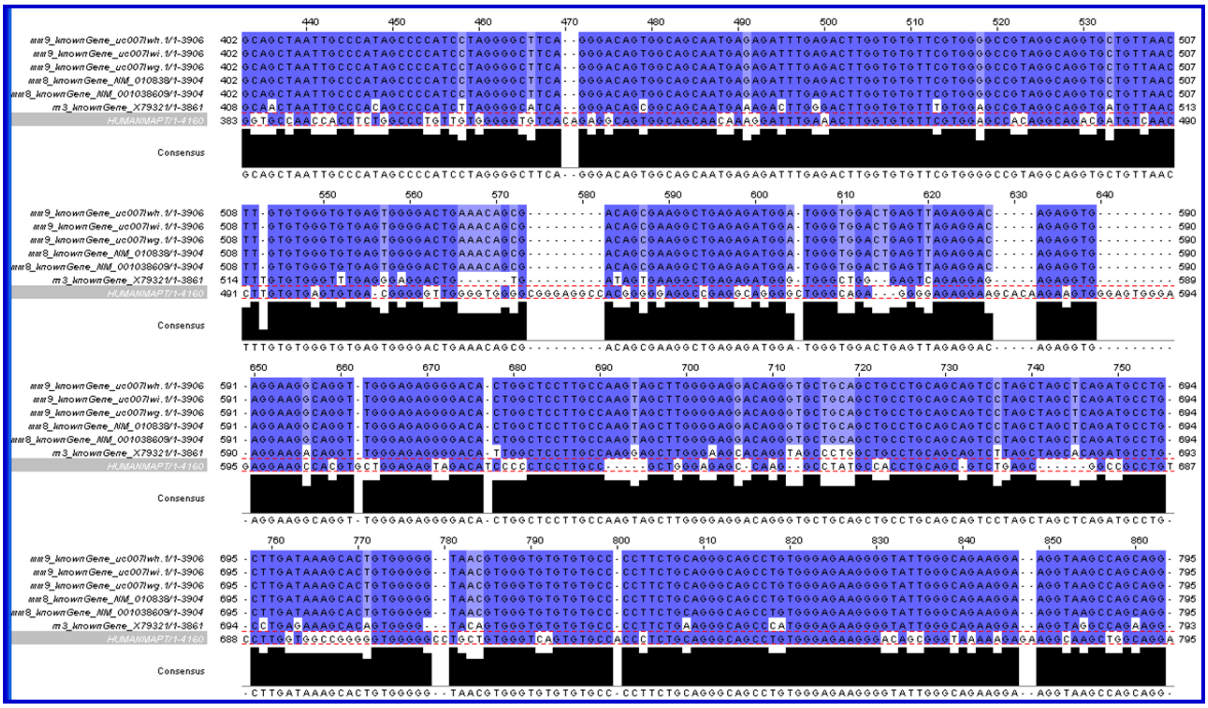
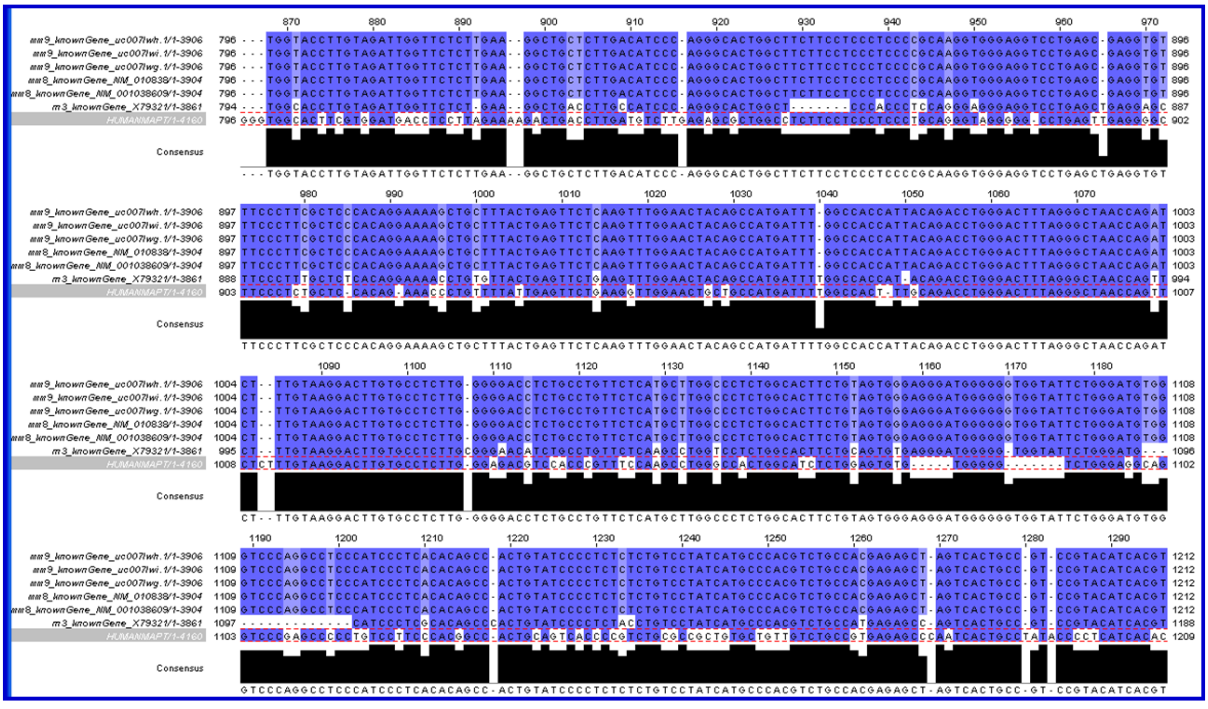
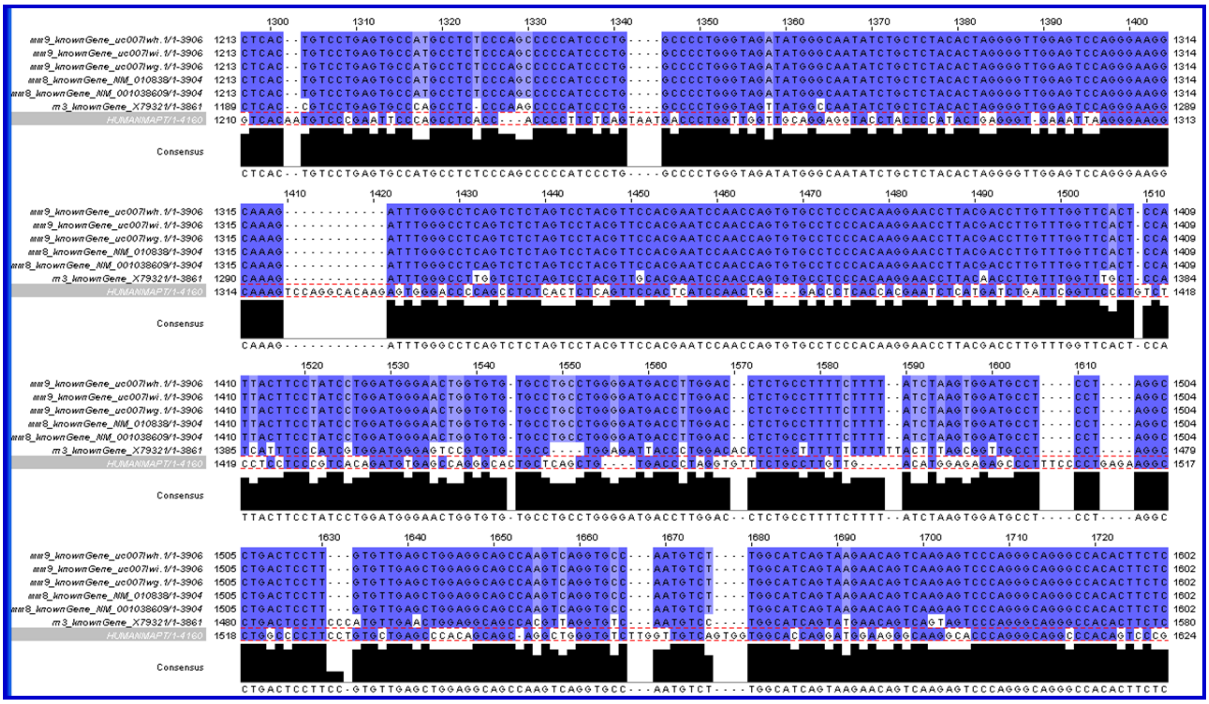


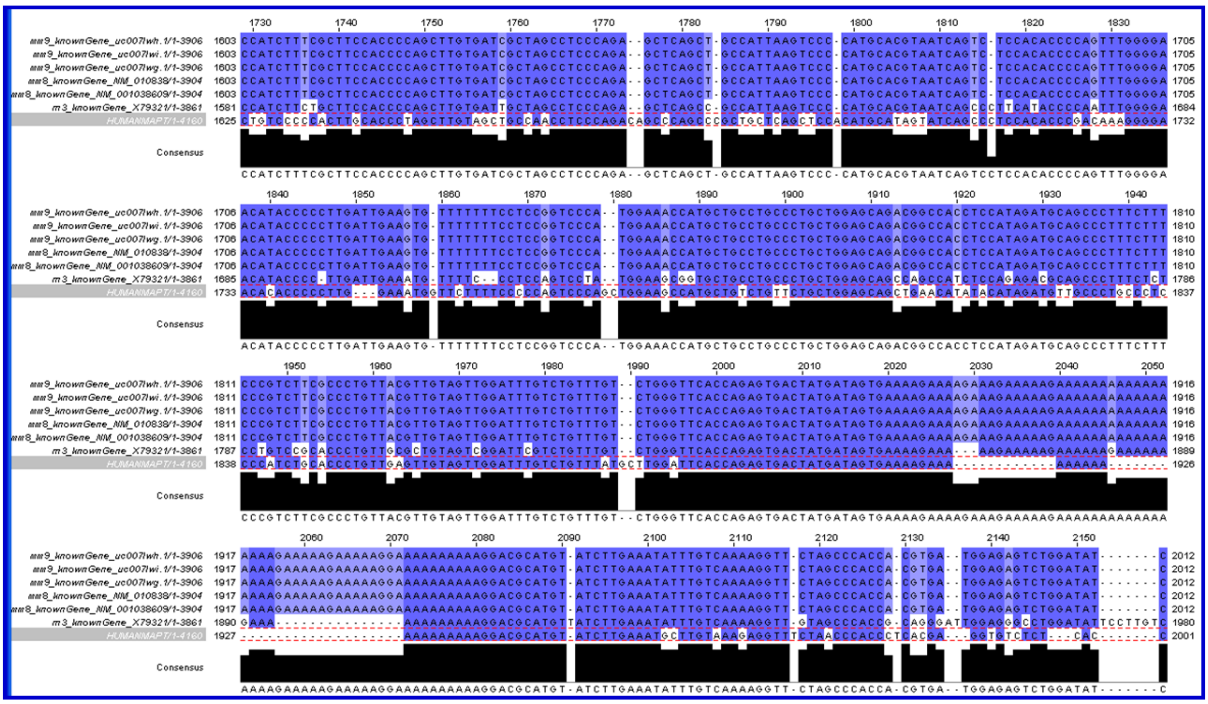
Figure 5.2.1a Scheme of the potential miRNAs binding sites on MAPT 3'UTR Fragment V. Schematic representation of putative miRNAs binding sites, present on MAPT 3'UTR Fragment V, according to the computational prediction of PITA, Targetscan and Targetprofiler.

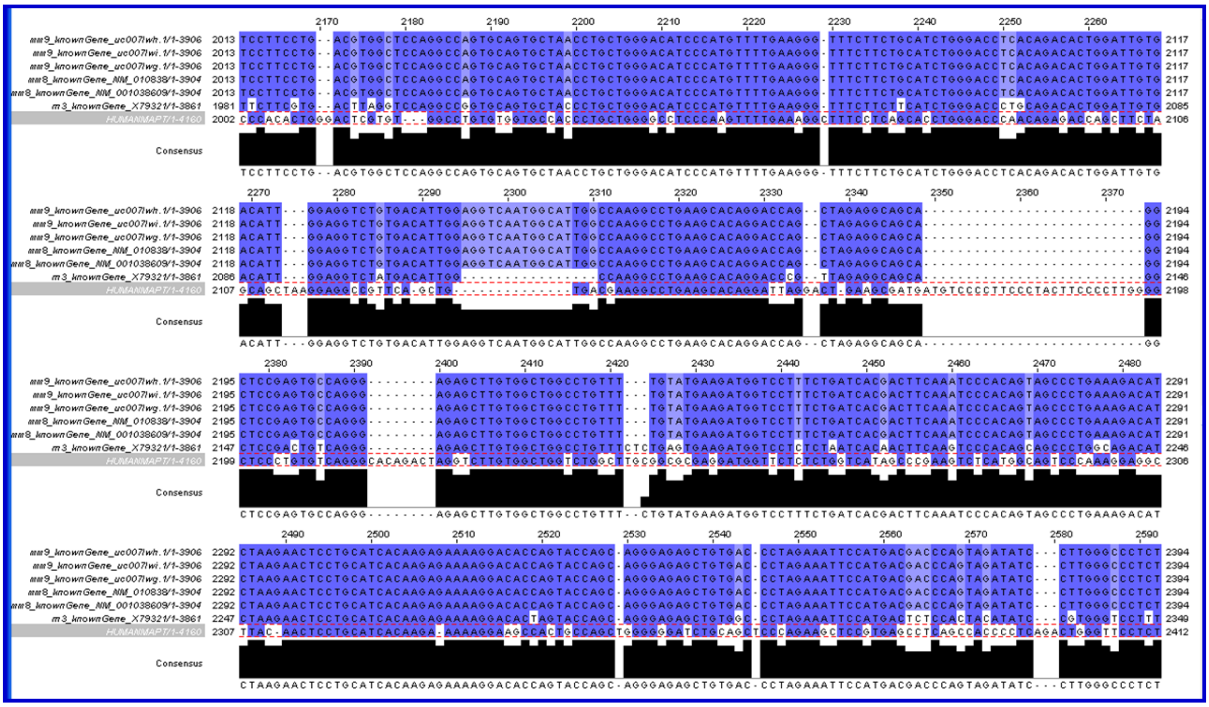


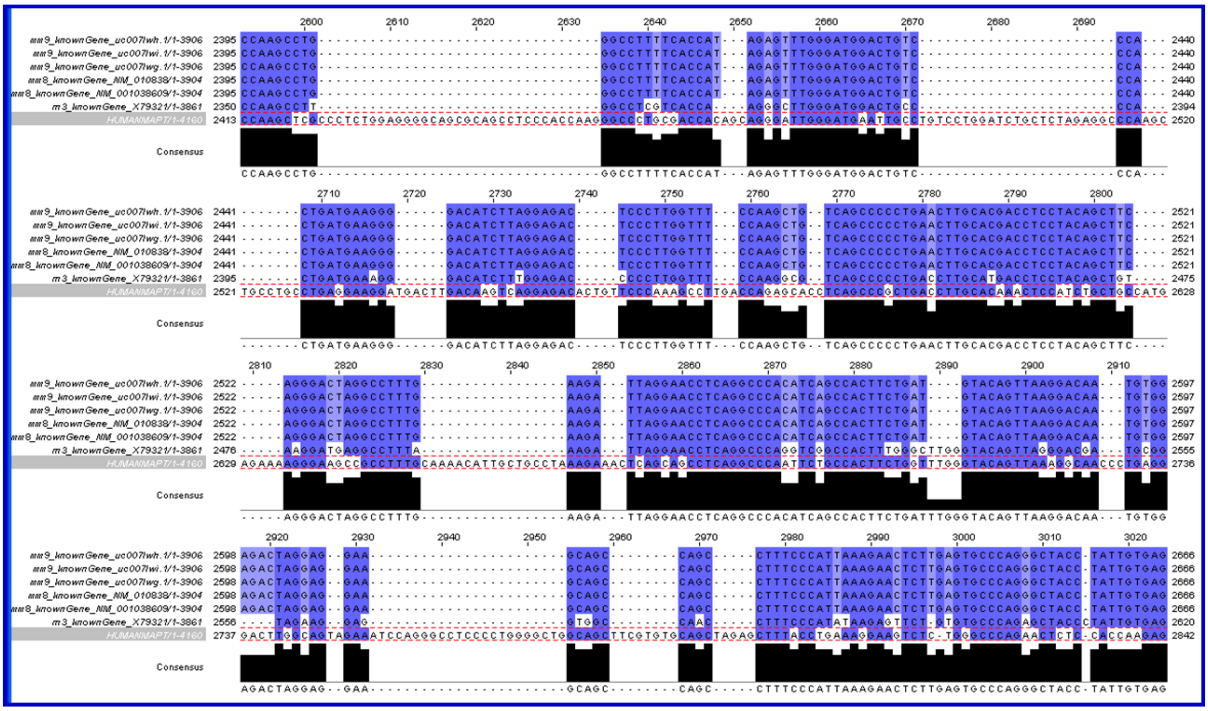


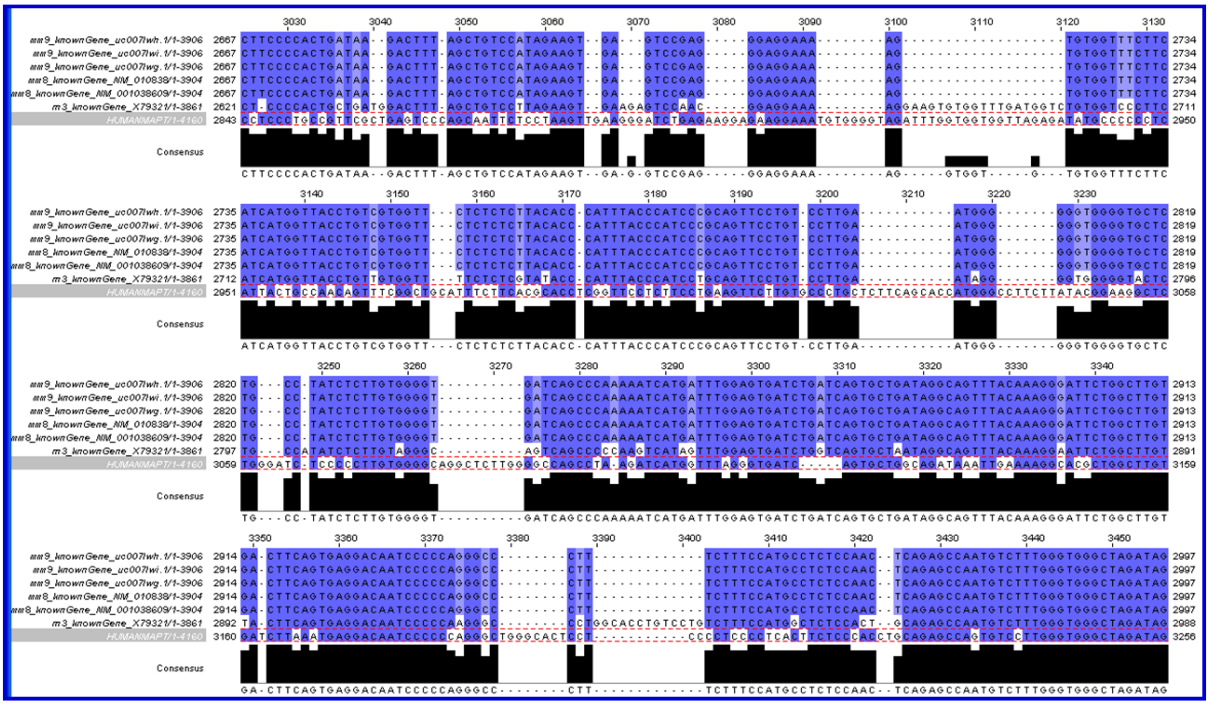


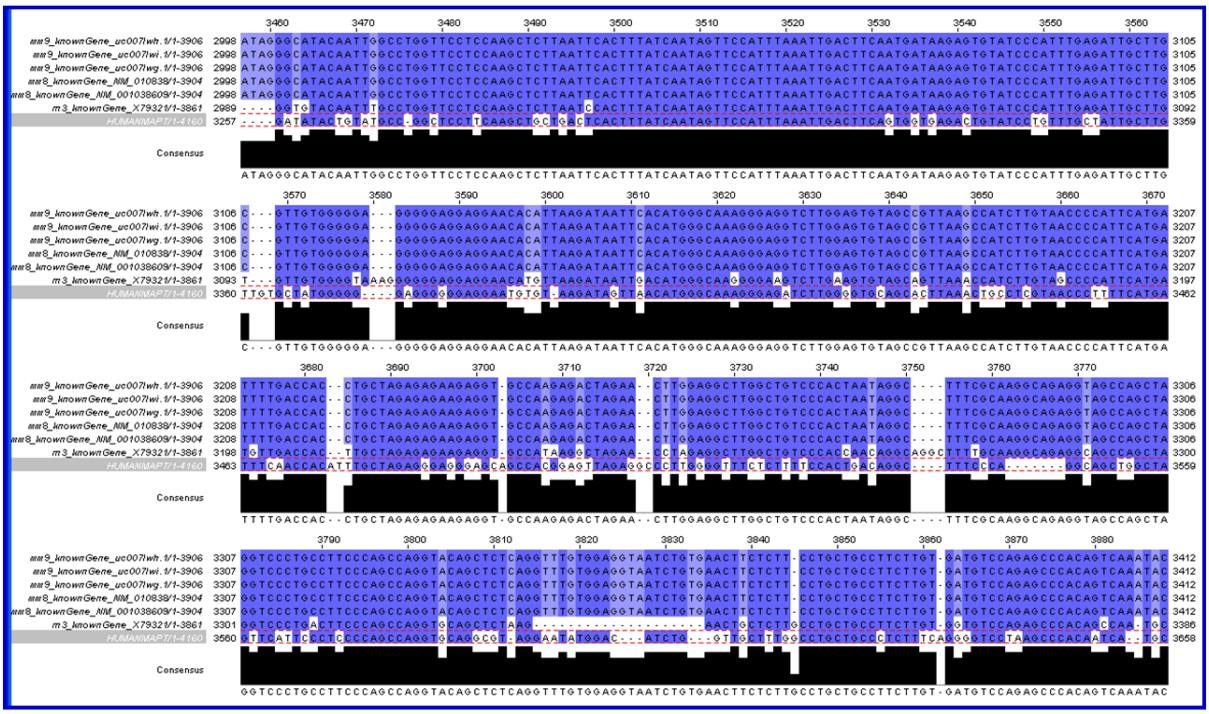












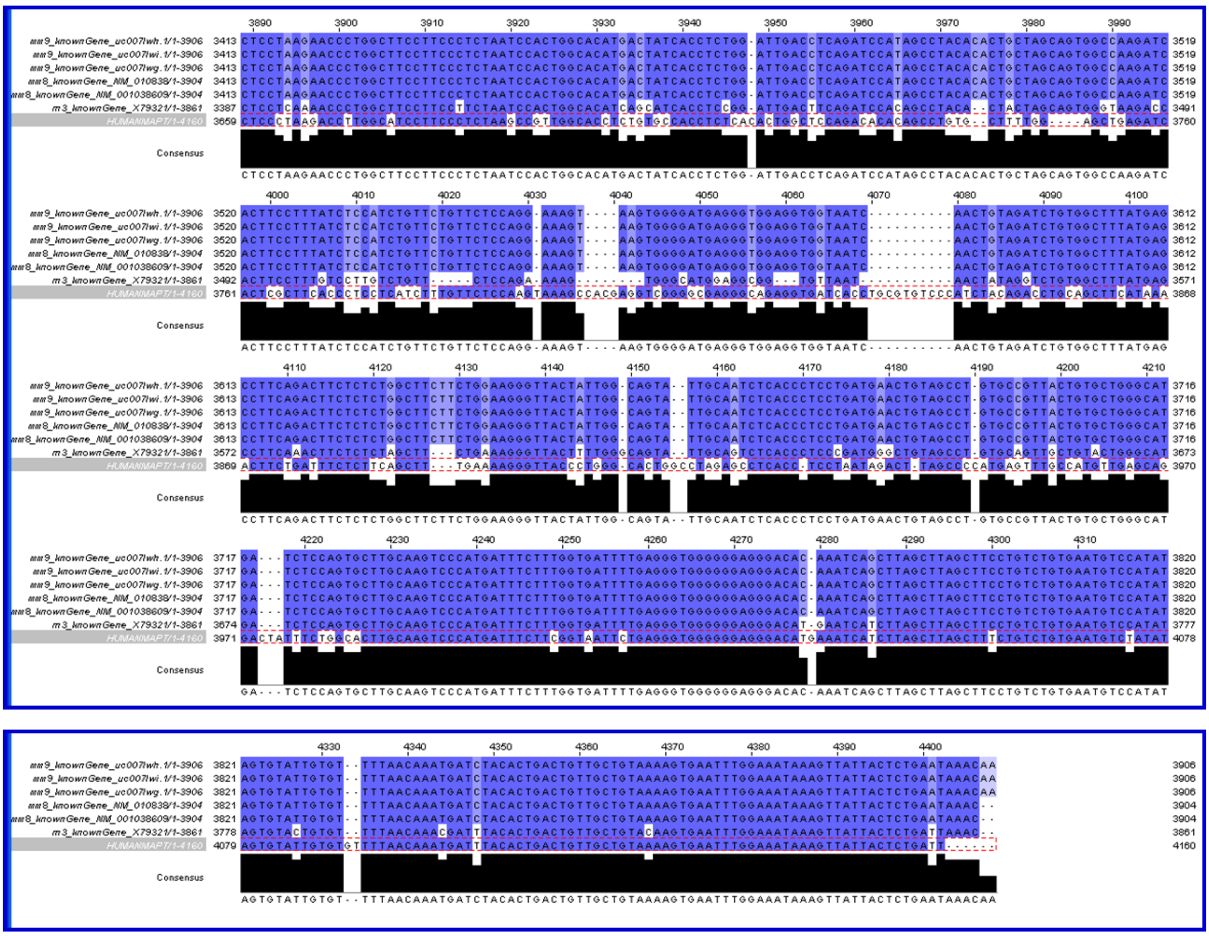


Figure 5.2.1b Conservation analysis of MAPT 3'UTR among human, mouse and rat. Multiple alignment of MAPT 3'UTR sequences among human, mouse and rat species performed by ClustalW and graphically represented with Jalview. Black squares below the alignment and dark blue color on the sequences indicate the 100% conservation between human, mouse and rat.

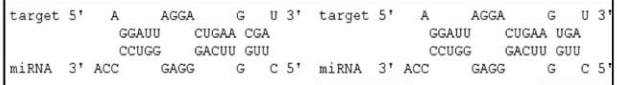
5.2.2 MAPT 3'UTR Fragment V Analysis of SNPs Located on miRNAs Binding Sites

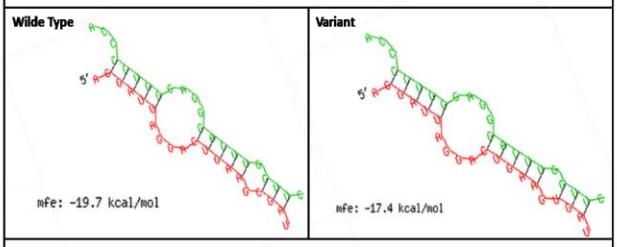
In the present analysis twenty-two different SNPs or small insertion and deletion were identified in the Fragment V of MAPT 3'UTR, using dbSNPs database. Seven of which were located in putative miRNAs binding sites. RNAhybrid software was used to calculate the free binding energy (ΔG) of miRNA-tagert interactions, in the presence of the common or variant sequence. As reported previously the difference between the free binding energy of the common and variant allele was calculated as variation (ΔΔG), according to the method developed by Saba and colleagues (Saba *et al.* 2014). Based on the variation values (ΔΔG), SNPs that increase miRNA binding have a positive variation (ΔΔG>0 kcal/mol), SNPs that reduce miRNA binding have a negative variation (ΔΔG<0 kcal/mol) and neutral SNPs that have no effect on miRNA binding have a variation equal to zero (ΔΔG=0 kcal/mol) (Saba *et al*. 2014). As reported in the **Figure 5.2.2** four SNPs caused an enhanced miRNA binding on Fragment V of MAPT 3'UTR, one is located on miR-659-3p binding site (**Figure 5.2.2 B**), two are located on the second binding of miR-608 (**Figure 5.2.2 D** and **E**) and the fourth one is present on the third binding of miR-608 (**Figure 5.2.2 J**). All the other SNPs overlapping miRNAs interaction regions induced a decreased miRNA binding.

Target: MAPT 3'UTR-Fragment 5
miRNA: miR-659 Binding Site

A

SNP: rs552535918, Alleles C/T



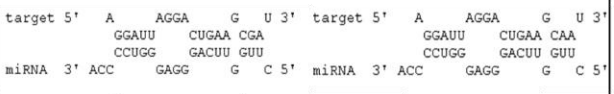


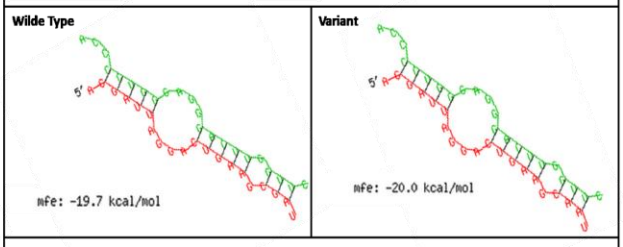
ΔΔG= -2,3

Target: MAPT 3'UTR-Fragment 5
miRNA: miR-659 Binding Site

B

SNP: rs16940802, Alleles A/G



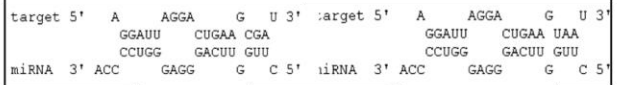


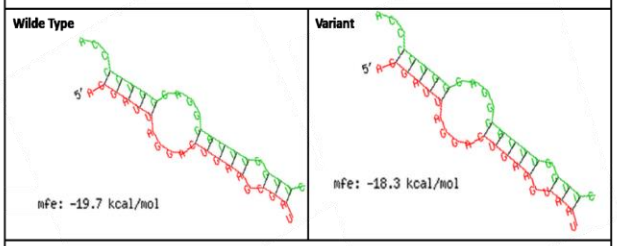
ΔΔG= 0,3

Target: MAPT 3'UTR-Fragment 5
miRNA: miR-659 Binding Site

C

2 SNPs: rs552535918, Alleles C/T
rs16940802, Alleles A/G



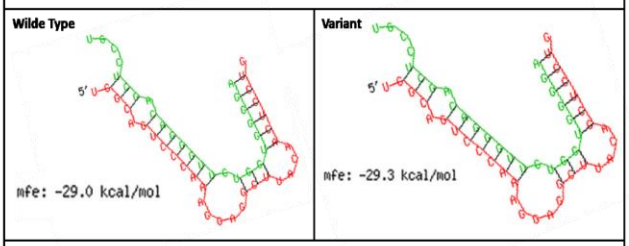


ΔΔG= -1,4

Target: MAPT 3'UTR-Fragment 5
miRNA: miR-608 II Binding Site

D

SNP: rs564290656, Alleles A/G

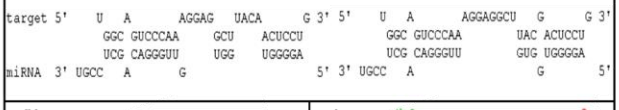


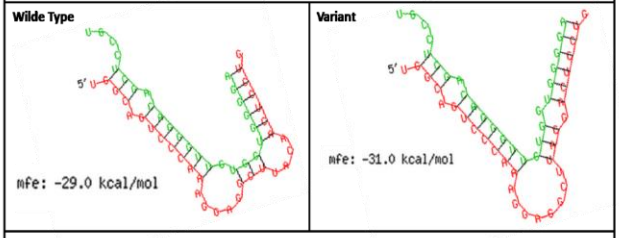
ΔΔG= 0,3

Target: MAPT 3'UTR-Fragment 5
miRNA: miR-608 II Binding Site

E

SNP: rs544368494, Alleles A/G



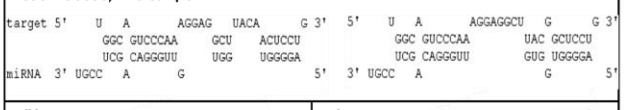


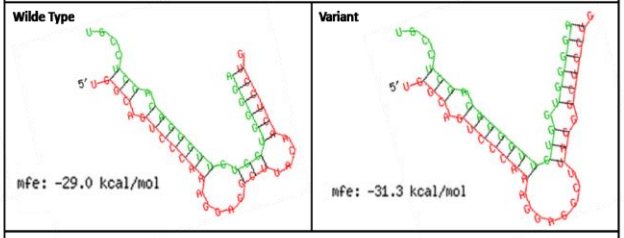
ΔΔG= 2

Target: MAPT 3'UTR-Fragment 5
miRNA: miR-608 II Binding Site

F

2 SNPs: rs564290656, Alleles A/G
rs564290656, Alleles A/G





ΔΔG=

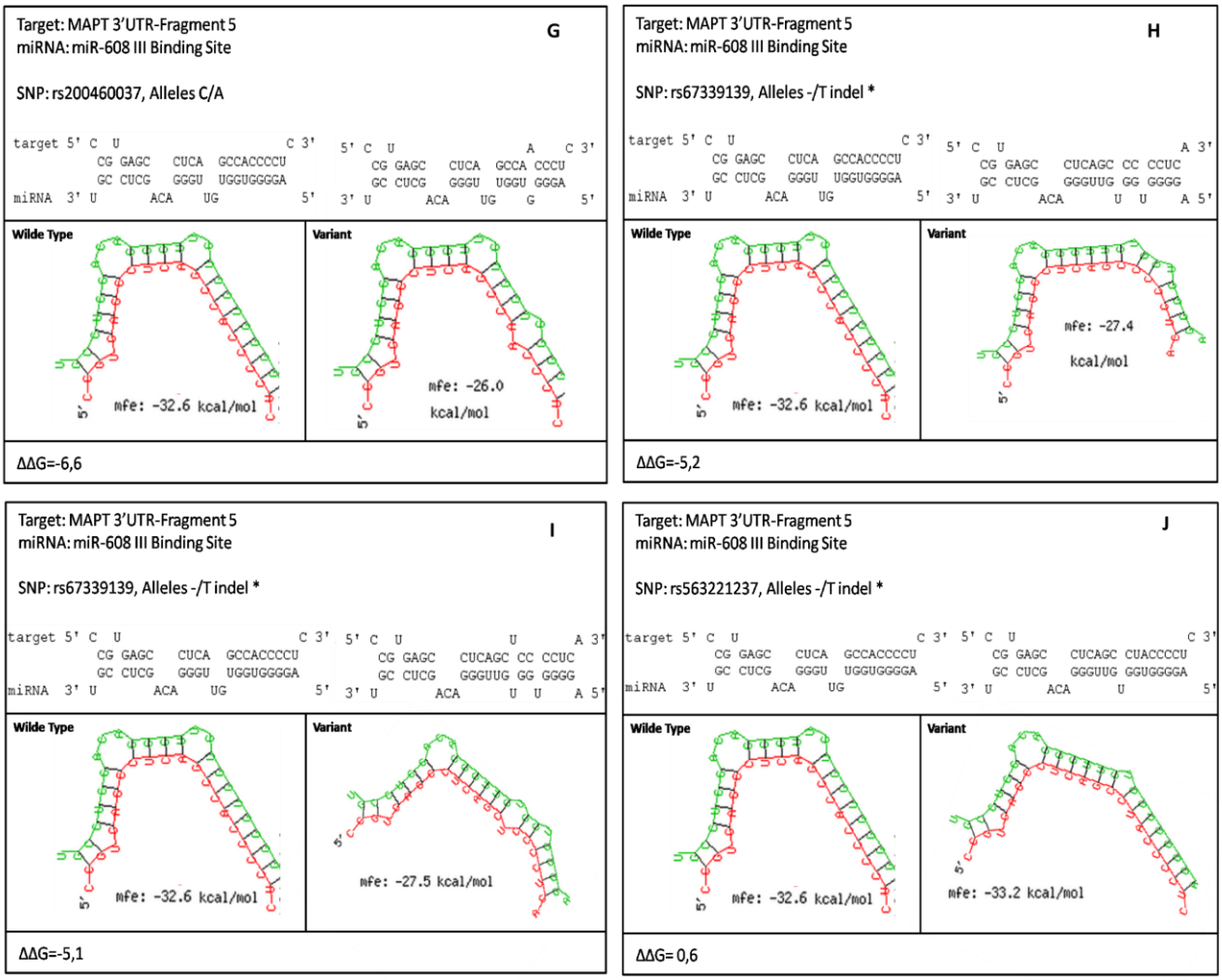


Figure 5.2.2 SNPs overlapping miRNA binding sites analysis for Fragment V of MAPT 3'UTR region. Representation of the miRNA-MAPT mRNA functional characterization technique following the method reported by Saba and colleagues (Saba *et al*. 2014). (* The sequence used as wild type in the figures **H, I** and **J** derived from USCS Genome Browser database (http://genome.ucsc.edu/) and represents the variant with CA bases, without the insertion of T allele).

5.2.3 MicroRNA Capture Affinity Technology (miR-CATCH)

Due to the MAPT 3'UTR length (4160 bp), the process of computational miRNAs target recognition is challenging and complicated for the entire region. Moreover, as reported for GRN 3'UTR, the high false-positive rate, and the presence of multiple algorithms that lead to different outputs, increase the inconsistency of bioinformatic prediction. For all these reasons, the miR-CATCH approach was used to analyze the subset of miRNAs that are targeting MAPT transcripts and can be isolated in physiological conditions. The same neuroblastoma cell lines used for the GRN miR-CATCH was applied for the pull down of MAPT transcripts. Therefore KELLY cell line was used for the optimization of the miR-CATCH protocol and the validation of the isolation method of MAPT mRNAs. Subsequently, the miR-CATCH strategy was used to investigate the regulation played by miRNAs on MAPT mRNAs in the selected neuroblastoma cell lines: KELLY, SH-SY5Y and SK-N-BE.

5.2.3.1 MAPT Biotinylated DNA Oligonucleotide Design

The secondary structures of all the 6 MAPT isoforms reported to be present in adult brain were modeled using M-Fold web server. Two of the most thermodynamically stable structures for each MAPT isoforms were elaborated and analyzed with UGENE program. This software allows the identification of the most probable single strand regions present on these transcripts. One exposed single strand region localized between bases 852 and 871, according to the longest MAPT transcript, was used as a target sequence to design the MAPT DNA capture oligonucleotide biotinylated at the 5'end, as reported in the **Figure 5.2.3.1**. This region of 20 bp, was analyzed with Basic Local Alignment Search Tool (BLAST), obtaining an exclusive 100% of total coverage to MAPT mRNA isoforms. However, BLAST showed a 17 bp similarity with the homo sapiens visual system homeobox 1 (VSX1) transcript variant 2 mRNA. An antisense DNA capture oligonucleotide biotinylated at the 5'end was designed against the single strand region found on MAPT mRNAs sequences (5'-GGG TGG TGT CTT TGG AGC GG -3'). Due to the complexity of the miR-CATCH protocol for all the six different isoforms of MAPT transcripts and the complicated identification of the single strand region for all the MAPT transcripts, the mismatch DNA oligonucleotide containing three different bases was not designed. However, for the validation of the MAPT mRNAs enrichment in biological triplicates, the DNA scrambled oligonucleotide biotinylated at the 5' end (5'-ATA TAT TAG ATT GCG TAT AAT TAG G-3'), was used as specific control as reported previously for the GRN miR-CATCH experiments.

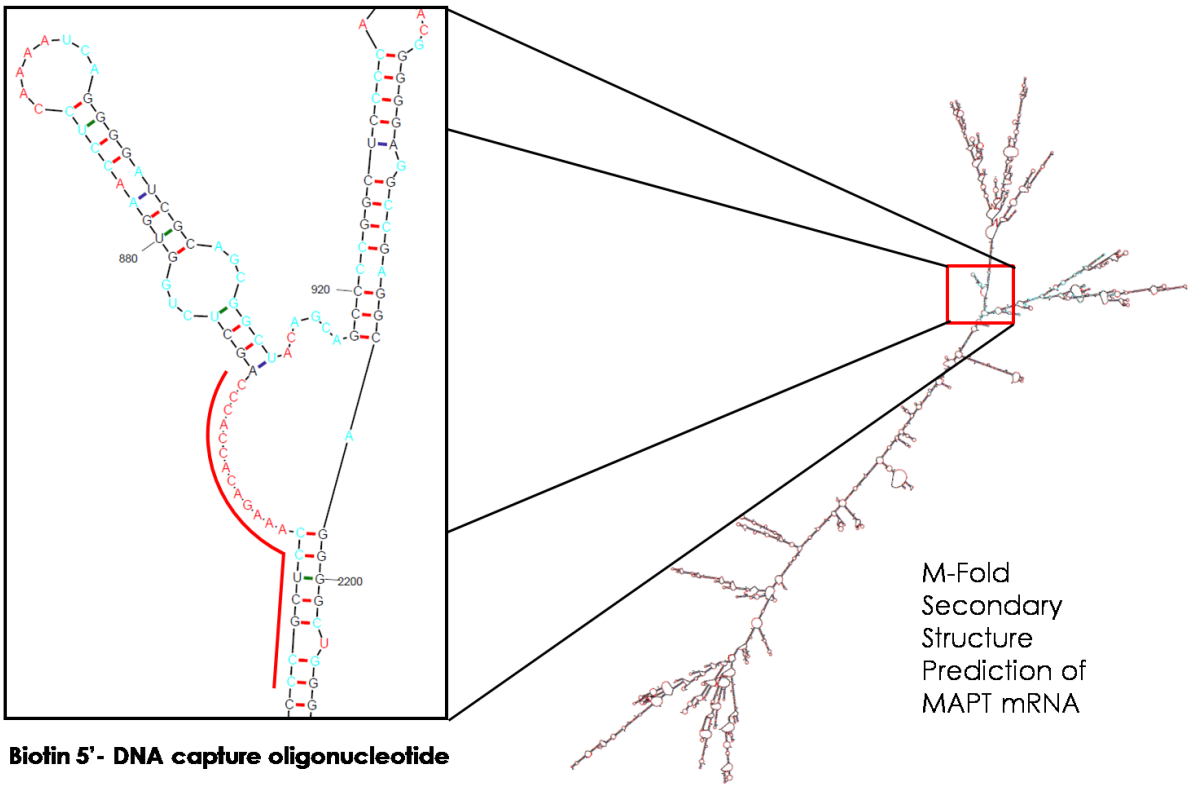


Figure 5.2.3.1 Design of MAPT mRNA Capture Oligonucleotide. Secondary structure of MAPT mRNA was elaborated with M-Fold. The exposed single strand region located between bases 852 and 871 of the transcript was used as target.*

*Optimization of the miR-CATCH technique for the isolation of GRN and MAPT mRNAs was done in collaboration with Dr. Sebastian Vencken, at the laboratory of Respiratory Research at the Royal College of Surgeons in Ireland, Beaumont Hospital

SINGLE STRAND REGION :	5’- CCGCTCCAAAGACACCACCC-3’
REVERSE AND COMPLEMENT SEQUENCE:	5’- GGGTGGTGTCTTTGGAGCGG-3’
OLIGO SEQUENCE:	Biotin 5’-GGG TGG TGT CTT TGG AGC GG -3’
LENGTH:	20
GC CONTENT:	65%
MELT TEMP:	74,9°C
HAIRPINS TM:	19°C
HOMO DIMER ANALYSIS:	≥-3,61 Kcal/mole

Table 5.2.3.1 Sequence and characteristics of the designed MAPT mRNAs Capture Oligonucleotide.

5.2.3.2 Validation of MAPTmRNA:miRNA Isolation

In order to validate the efficacy of MAPT transcripts isolation, the first experiment was performed with KELLY cell lysate, using MAPT capture oligonucleotide. Following the miR-CATCH protocol, the eluted samples were analyzed with Real Time PCR. Specific primers quantified the total amount of MAPT transcripts, due to the amplification of a product of 150 bp. Whereas GAPDH expression was used to normalize the MAPT transcripts quantification. Levels of MAPT mRNAs measured in the capture sample were compared to the levels obtained from the same cell lysates that did not undergo the capture procedure. Using the $2^{-\Delta\Delta Ct}$ method there was an enrichment of 28,84 fold compared the total RNA extracted from the same lysate of KELLY cells, as reported in the **Figure 5.2.3.2**. Since MAPT capture oligonucleotide showed complementarity for the visual system homeobox 1 (VSX1) transcript variant 2 mRNA, specific primers were designed for the detection of this transcript in the capture sample. Real Time PCR demonstrated no enrichments for VSX1 transcript in the MAPT capture samples, compared to the total RNA (data not shown). This result ruled out the possibility to obtain an off target effect due to the presence of contaminant transcripts in the capture samples.

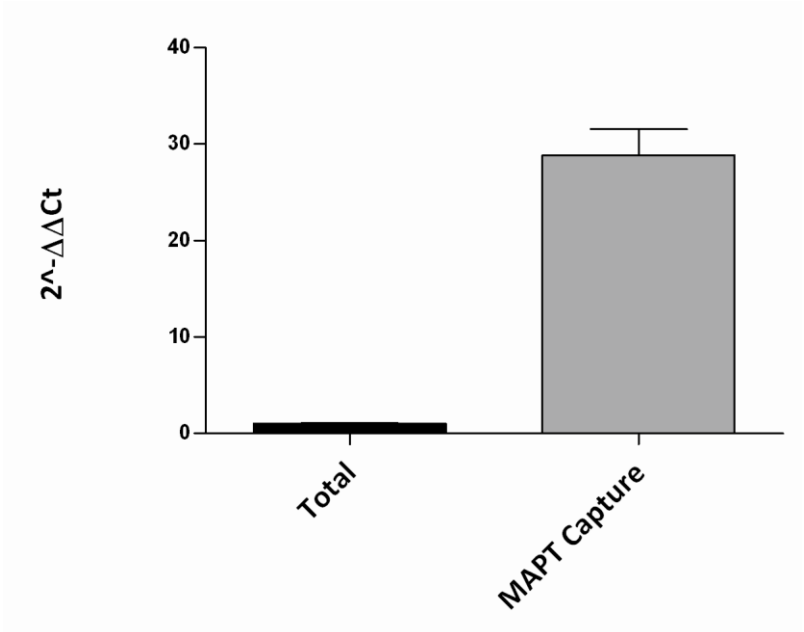


Figure 5.2.3.2 Validation of the MAPT mRNAs isolation. Representation of the qRT-PCR data, showing the fold enrichment level ($2^{-\Delta\Delta Ct}$) obtained with MAPT capture oligonucleotide

compared to the total RNA that did not undergo the miR-CATCH protocol. Expression of MAPT mRNAs was normalized against GAPDH expression.Representative data of one biological experiment and three technical replicates (Mean ±SD).

5.2.3.3 MAPT mRNAs enrichment in KELLY, SH-SY5Y and SK-N-BE cell lines

After the validation of miR-CATCH protocol with KELLY cell line, the isolation of MAPT transcripts was performed in biological triplicates for three different neuroblastoma cell lines: KELLY, SH-SY5Y and SK-N-BE. As negative controls, scrambled oligonucleotides biotinylated at the 5'end, were used for the miR-CATCH experiments. By Real time PCR, MAPT mRNAs expression was quantified, and normalized using GAPDH mRNA expression. The MAPT mRNAs quantity presents in the capture samples of each biological experiment was compared to that present in the scrambled controls using $2^{-\Delta\Delta Ct}$ method. In particular 23 fold enrichment of MAPT mRNAs was obtained in KELLY cell line, whereas 2,5 fold enrichment was found in SH-SY5Y cell line and 4 fold enrichment for SK-N-BE cell line, compared to scrambled control (**Figure 5.2.3.3).**

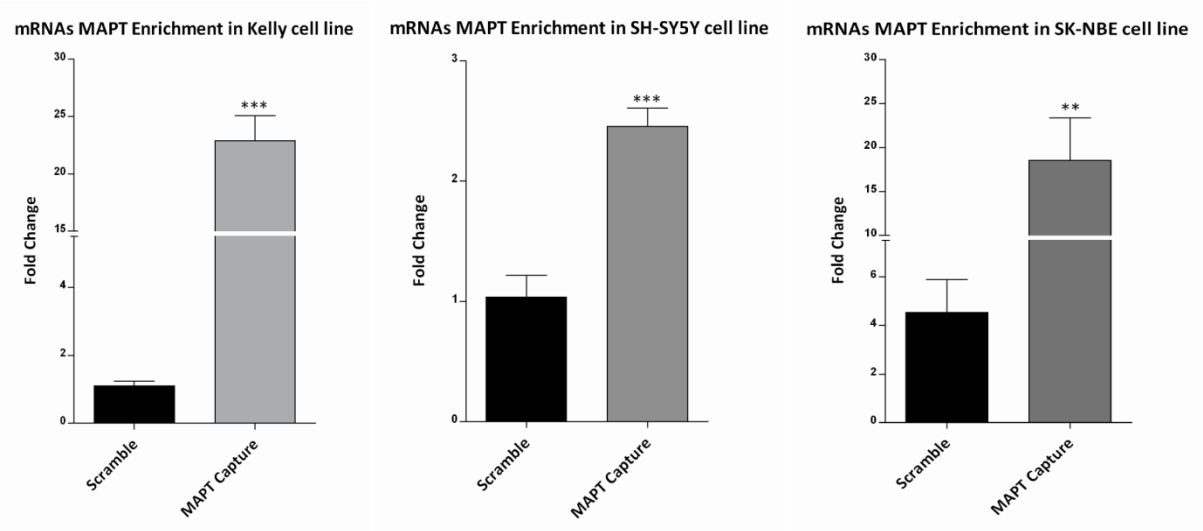


Figure 5.2.3.3 MAPT mRNAs enrichment in different neuroblastoma cell lines. Representation of the fold enrichment ($2^{-\Delta\Delta Ct}$) values of MAPT mRNAs compared to specific scrambled controls. Data were normalized with GAPDH expression. Mean ± SEM of three biological replicates is shown in the figure (** p<0,001; *** p<0,0001 vs. scrambled control).

5.2.3.4 Selected miRNAs enrichment on MAPT Capture Samples in KELLY, SH-SY5Y and SK-N-BE cell lines

The miR-CATCH MAPT samples were further analyzed for the presence of the putative miRNAs of interest (miR-659-3p, miR-939-5p, miR-608 and miR-615-5p). TaqMan Real Time PCR primers were used for the specific quantification of these miRNAs in the MAPT capture samples compared to the values measured on the scrambled controls, using the $2^{-\Delta Ct}$ method. **Figure 5.2.3.4.1** and **Figure 5.2.3.4.2** represent the mean level of the specific miRNAs enrichment derived from biological triplicates for each different cell line. miR-659-3p shows an enrichment of 474,5 times in MAPT capture samples derived from KELLY cell line (**Figure 5.2.3.4.1 A**), 162620 times and 120319 times in MAPT capture samples derived from SH-SY5Y and SK-N-BE cell lines, respectively (**Figure 5.2.3.4.2 A and B**). MiR-939-5p was found highly enriched in all the three biological experiments performed in KELLY cell line (**Figure 5.2.3.4.1 B),** in SH-SY5Y and SK-NBE cell lines (**Figure 5.2.3.4.2 A and B**).MiR-608 and miR-615-5p were also quantified by TaqMan qRT-PCR in MAPT capture samples, however these miRNAs were not found enriched in all the cell lines analyzed, probably due to the lack of their basal expression in these neuroblastoma cell lines in physiological conditions (data not shown). The expression of miR-323 was used as negative control in KELLY cell line. miR-323 was not enriched in the capture samples compared to scrambled controls (**Figure 5.2.3.4.1 C**).

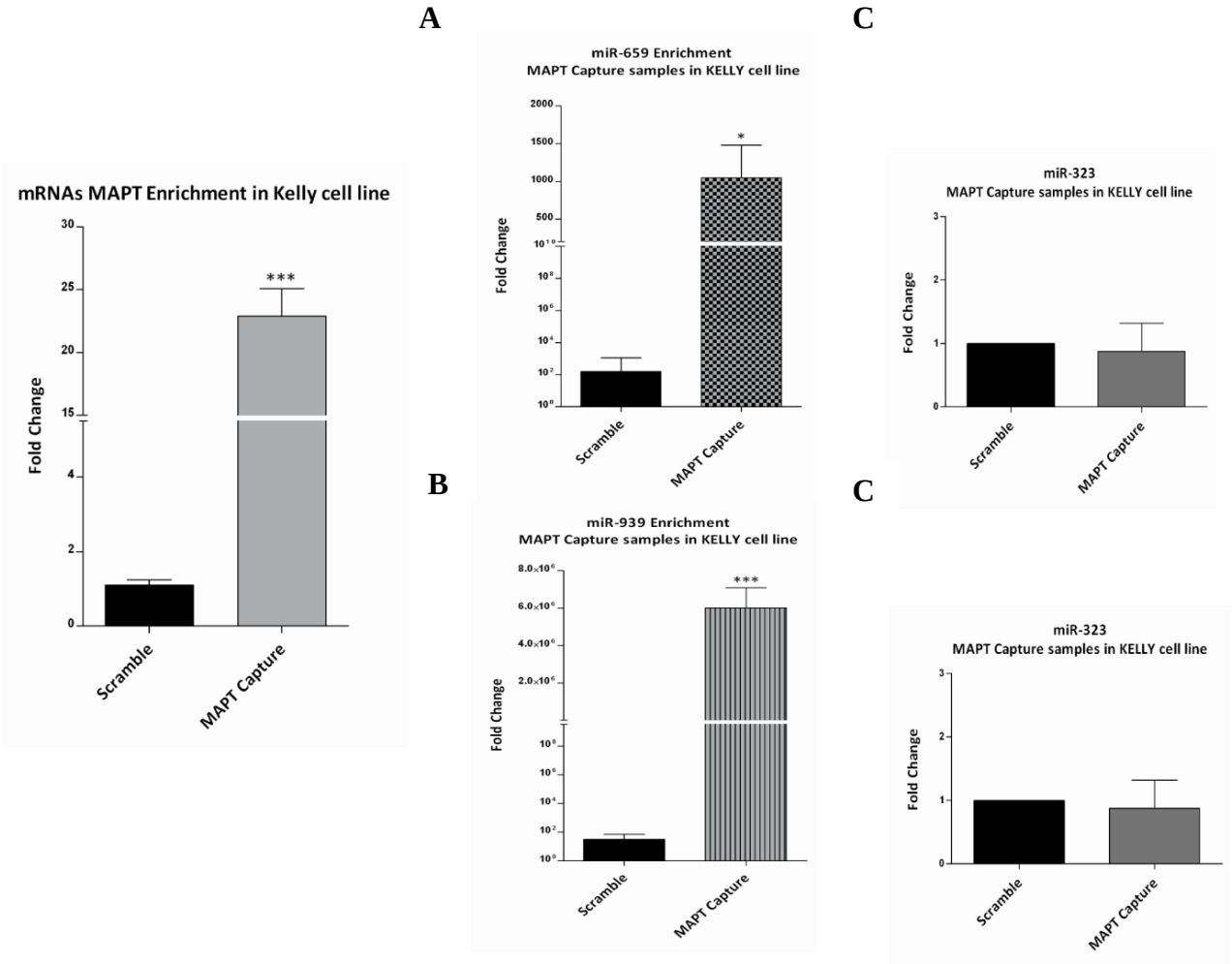


Figure 5.2.3.4.1 Selected miRNAs enrichment in KELLY cell line. Representation of miR-659-3p **A)** and miR-939-5p **B)** significant enrichment in MAPT capture samples compared to scrambled controls. Panels **C)** represent the negative control miR-323, that shows no enrichment in the capture samples. miRNAs expression was represented as fold enrichment value ($2^{-\Delta Ct}$).**A)** and **B)** Mean±SEM of three different biological experiments (*P<0,05; *** P<0,0001). **C)** Representative figures of two biological samples with three technical replicates.

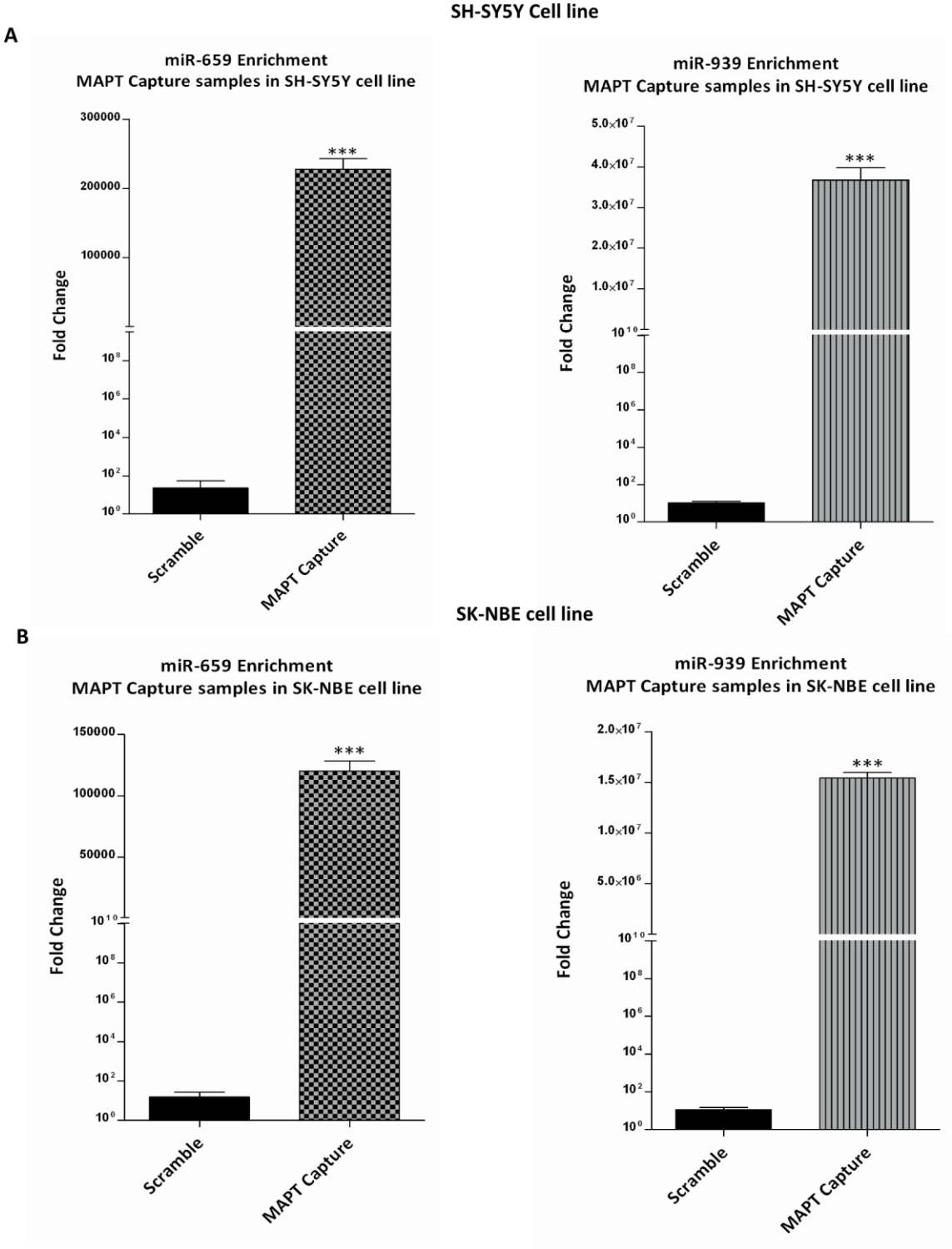


Figure 5.2.3.4.2 Selected miRNAs enrichment in SH-SY5Y and SK-NBE cell lines. A) Representation of miR-659-3p and miR-939-5p enrichment found in MAPT capture samples compared to scrambled controls in SH-SY5Y samples. **B)** Graphs representing the enrichment of miR-659-3p and miR-939-5p compared to the scrambled controls in SK-NBE cell line. miRNAs expression was represented as fold enrichment value ($2^{-\Delta Ct}$).Mean ± SEM of three different biological experiments (*** P < 0,0001).

5.2.4 Luciferase Assays using reporter vectors of the MAPT 3'UTR in HeLa cells

The regulation of our miRNAs of interest was further investigated using luciferase reporter plasmid vectors and over-expressing plasmids for the putative miRNAs. The miR-CATCH experiments showed strong enrichments of miR-659-3p and miR-939-5p in MAPT capture samples derived from all the analyzed neuroblastoma cell lines. However, according to the bioinformatic predictions, miR-939-5p could also bind the coding region of MAPT mRNAs. Luciferase assays were used to analyze miR-659-3p and miR-608 regulation on MAPT 3'UTR. These miRNAs were predicted to bind Fragment V of MAPT 3'UTR (**Figure 5.2.1a**). Luciferase experiments were performed at 24h and 48h from the transfection day. The over-expressing plasmids for miR-608 and miR-659-3p were co-trasfected with luciferase reporter vectors containing: the full length MAPT 3'UTR (pmiRGLO-FullMAPT), MAPT 3'UTR Fragment V (pmiRGLO-MAPTV) and MAPT 3'UTR Fragment II (pmiRGLO-MAPTII) that does not have any potential binding sites for miR-608 and one putative binding for miR-659-3p. The levels of luciferase activity measured in the presence of the miRNAs were compared to the levels obtained from samples transfected with the empty over-expressing plasmid. After 24h there was a significant luciferase down-regulation using miR-608 and miR-659-3p co-transfected with the full length MAPT 3'UTR and with the Fragment V. In particular a strong luciferase reduction, up to 50% and 46%, was obtained with the co-transfection of Fragment V with miR-608 and miR-659-3p respectively, as reported by the **Figure5.2.4 (A)**. The co-transfection using the full length MAPT 3'UTR produced a significant down-regulation of 30% with miR-608 and 22% with miR-659-3p, compared to the empty control. Whereas the co-transfection of Fragment II with the over-expressing plasmids for miR-608 and miR-659-3p did not show any luciferase down-regulation. At 48h there was a significant increase in the reduction of luciferase due to the over-expression of miR-659-3p with the presence of the full length MAPT 3'UTR, that reached the 49%, as represented in the **Figure5.2.4 (B)**, while miR-608 caused a luciferase reduction of 36%. At 48h even the co-transfection of Fragment V with miR-659-3p led to a significant down-regulation of 35%, while miR-608 produced a small and not significant decrease of 17% compared to the empty controls. At 48h there was a decrease of luciferase in the co-transfection of Fragment II, with a major effect played by the over-expression of miR-659 (up to a 30% decrease).

Indeed Fragment II contains only one putative binding site for miR-659. All these experiments derived from three or four biological replicates (Mean ± SEM). The experiments with fragment II at 48h were performed only two times and the observed luciferase reduction was not significant. Further repetitions would give more information about the regulation of these miRNAs on Fragment II and the dynamic of their reduction.

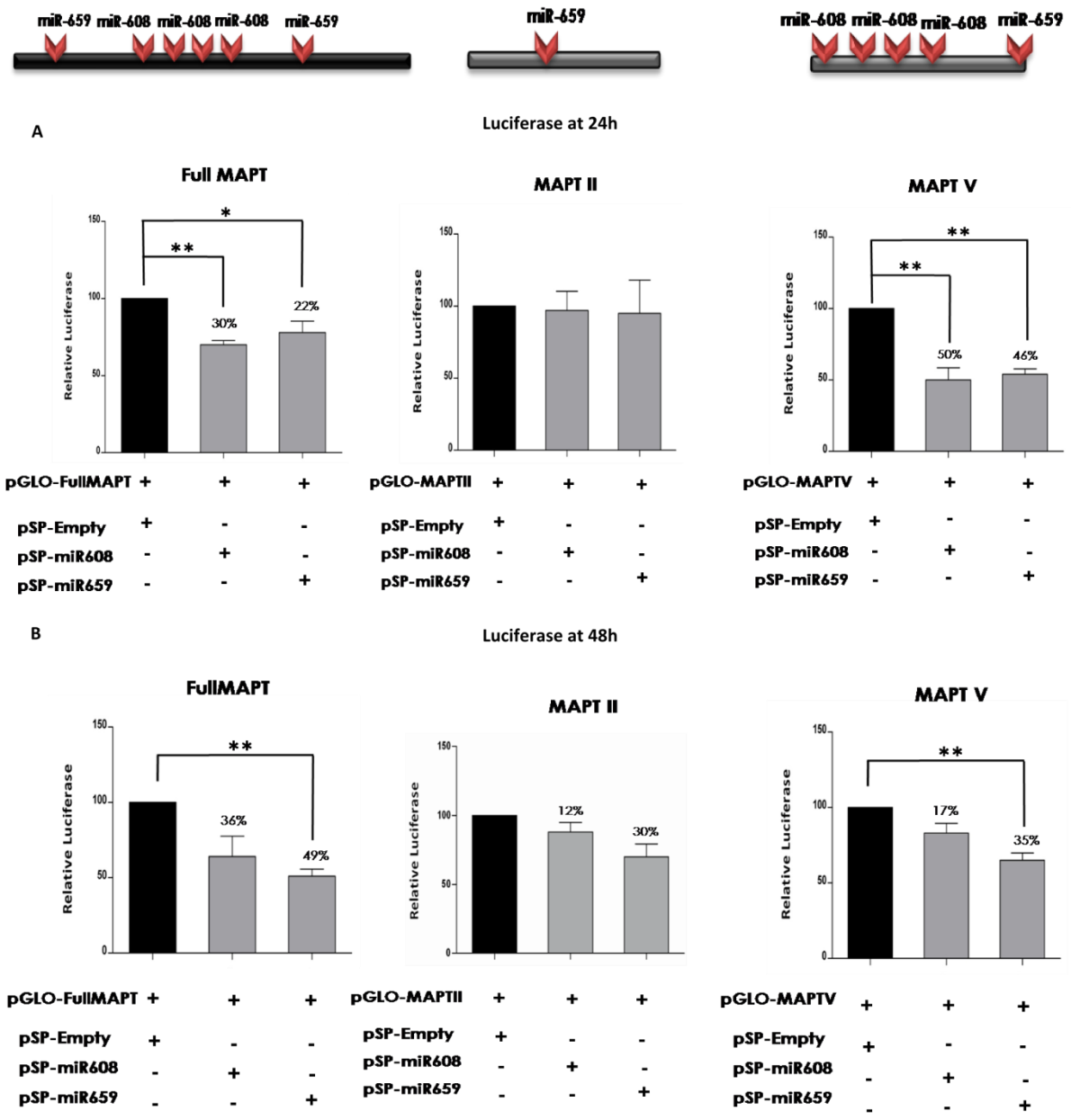


Figure 5.2.4 Luciferase assays of reporter plasmid of MAPT 3'UTR and over-expressing vectors for miR-608 and miR-659-3p. A) Relative luciferase activity was measured in HeLa cells transiently co-transfected with pmiRGLO vector containing the full length MAPT 3'UTR or fragment II of MAPT 3'UTR or fragment V of MAPT 3'UTR and the over-expressing plasmids after 24h. Significant luciferase down-regulation in presence of miR-608 and miR-

659-3p compared to the empty control. Luciferase reduction was not observed with the co-transfection of the fragment II of MAPT 3'UTR **B)** Relative luciferase activity analyzed at 48h using the same constructs. Significant luciferase down-regulation with miR-659-3p and the full length MAPT 3'UTR or fragment V of MAPT 3'UTR. Firefly luciferase activity was normalized with *Renilla* luciferase activity. Percentages of luciferase reduction, reported above the graph bars, are related to negative control, empty control vector. Mean ± SEM of three biological replicates is shown in the figure. The 48h of fragment II of MAPT 3'UTR are representative of two biological replicate experiments (Mean ± SEM).(*p<0,05, ** P<0,001).*

*This work was performed in collaboration with Dr.Margherita Grasso at the laboratory of RNA Biology and Biotechnology in CIBIO.

5.2.5 Effect of Selected miRNAs Overexpression on Tau protein level in KELLY cell line

In order to analyze the effect of miRNAs over-expression on tau protein, western blot analyses were performed after the over-expression of the putative miRNAs of interest. KELLY cell line was transfected with miR-615-5p, miR-939-5p, miR-659-3p, miR-608 over-expressing plasmids, the empty control that does not contain the precursor region of any miRNAs (pSP-Empty). HPRT protein was detected with specific antibody and used to normalize tau expression in all the analyzed conditions. The expression of tau quantified in all the transfected samples was compared to the quantity observed in empty control transfection, representing the endogenous level of tau present in KELLY cell line at physiological condition. As represented in **Figure 5.2.5** there was a down-regulation of tau level with the transfection of miR-659-3p and miR-608 compared to the control. In particular, transfection with miR-659-3p led to 87,5% of tau expression and transfection with miR-608 caused 84,1% of tau expression. Whereas transfection of miR-615-5p and miR-939-5p did not reduce the level of tau protein compared to the empty control, there was a slightly increase of the protein maybe due to an indirect effect.

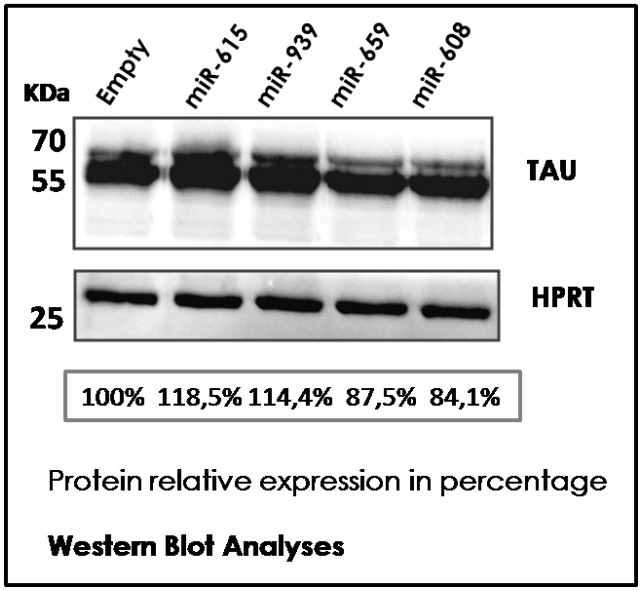


Figure 5.2.5 Effect of miRNAs over-expression on tau. The over-expression of miR-659-3p and miR-608 that show binding sites located on the MAPT 3'UTR, down-regulated the expression of tau protein, compared to control sample represented by the transfection with the empty vector. Percentages of tau protein expression reported below the image are calculated with empty plasmid comparison. Data normalized with HPRT protein expression. Representative western blot of an experiment repeated three times.

5.2.6 Effect of Selected miRNAs Overexpression on MAPT mRNAs level in KELLY cell line

The level of MAPT transcripts was quantified by Real Time PCR after the over-expression of all the miRNAs of interest. KELLY cell line was transiently transfected with the over-expressing plasmid for putative miRNAs panel and the backbone empty plasmid, used for miRNA cloning, as negative control. RNA was extracted after 48h from the transfection day and used for MAPT mRNAs quantification using qRT-PCR. MAPT mRNAs expression level was normalized with two housekeeping genes: the hypoxanthine phosphoribosyltransferase I (HPRTI) and the succinate dehydrogenase complex subunit A (SDHA). The fold changes were calculated with the $2^{-\Delta\Delta Ct}$ method, comparing the levels of MAPT mRNAs present in the samples transfected with miRNAs over-expressing plasmids, with the levels of MAPT mRNAs quantified in samples transfected with the negative control, the empty vector, as represented in the **Figure 5.2.6**. All the cells transfected with the putative miRNAs had a small and not significant or absent down-regulation of MAPT mRNAs level compared to negative control.

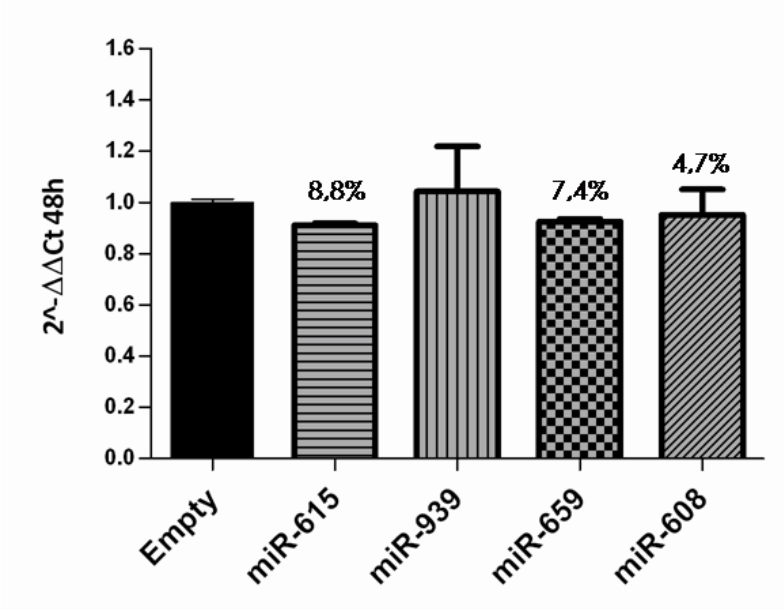


Figure 5.2.6 Effect of miRNAs over-expression on the MAPT mRNAs level. KELLY cells were transfected with the putative miRNAs and the empty control. RNA was extracted after 48h and analyzed using Real Time PCR for the detection of MAPT mRNAs and two specific normalizators, SDHA and HPRTI mRNAs. Representation of the fold changes calculated with the $2^{-\Delta\Delta Ct}$ method. Data derived from biological duplicate experiments (Mean ± SEM).

5.2.7 Effect of miRNAs Over-expression on MAPT mRNAs in polysomal and sub-polysomal compartments in KELLY cell line

Since the over-expression of miR-659-3p and miR-608 reduced tau protein level, but did not alter and degrade MAPT mRNAs level, KELLY cell line was used for the polysomal analysis to verify whether miR-659-3p and miR-608 regulate the level of tau by affecting the translation efficiency of MAPT mRNAs. KELLY cell line was transfected with miRNAs over-expressing vectors and empty controls, and after 48h, the mRNA-ribosomal complexes were separated through sucrose gradient centrifugation in polysomal and sub-polysomal fractions, containing the effectively translated transcripts and the untranslated mRNAs respectively. For each transfection condition, RNA was extracted from the polysomal and sub-polysomal compartments and used for Real Time PCR analysis, in order to quantify the MAPT mRNAs levels present in the polysomal and sub-polysomal parts. The expression of HPRTI mRNA was used as normalizator to calculate the $2^{-\Delta Ct}$ values and results are reported in **Figure 5.2.7**. The relative distributions of MAPT mRNAs in the two compartments were indicated as percentages calculated on the sum of the transcripts extracted from the two fractions, for each treatment and control. After the transfection of all the miRNAs of interest there was an increase in the amount of MAPT mRNAs quantified on the sub-polysomal parts, compared to the empty control. Indeed the experiments showed a trend toward a shift of MAPT mRNAs from the polysomal to the sub-polysomal compartment after the transfection with miRNAs compared to the empty control. The major increase of MAPT mRNAs were measured in the polysomal parts derived from samples transfected with miR-615-5p, miR-659-3p and miR-608. These results indicate that miR-659-3p and miR-608 could regulate the level of tau by affecting the translation efficiency of MAPT mRNAs. Whereas the effect of miR-615-5p over-expression on the translation of MAPT mRNAs could be potentially blocked by other cellular processes, since the expression of tau protein is not altered by miR-615-5p.

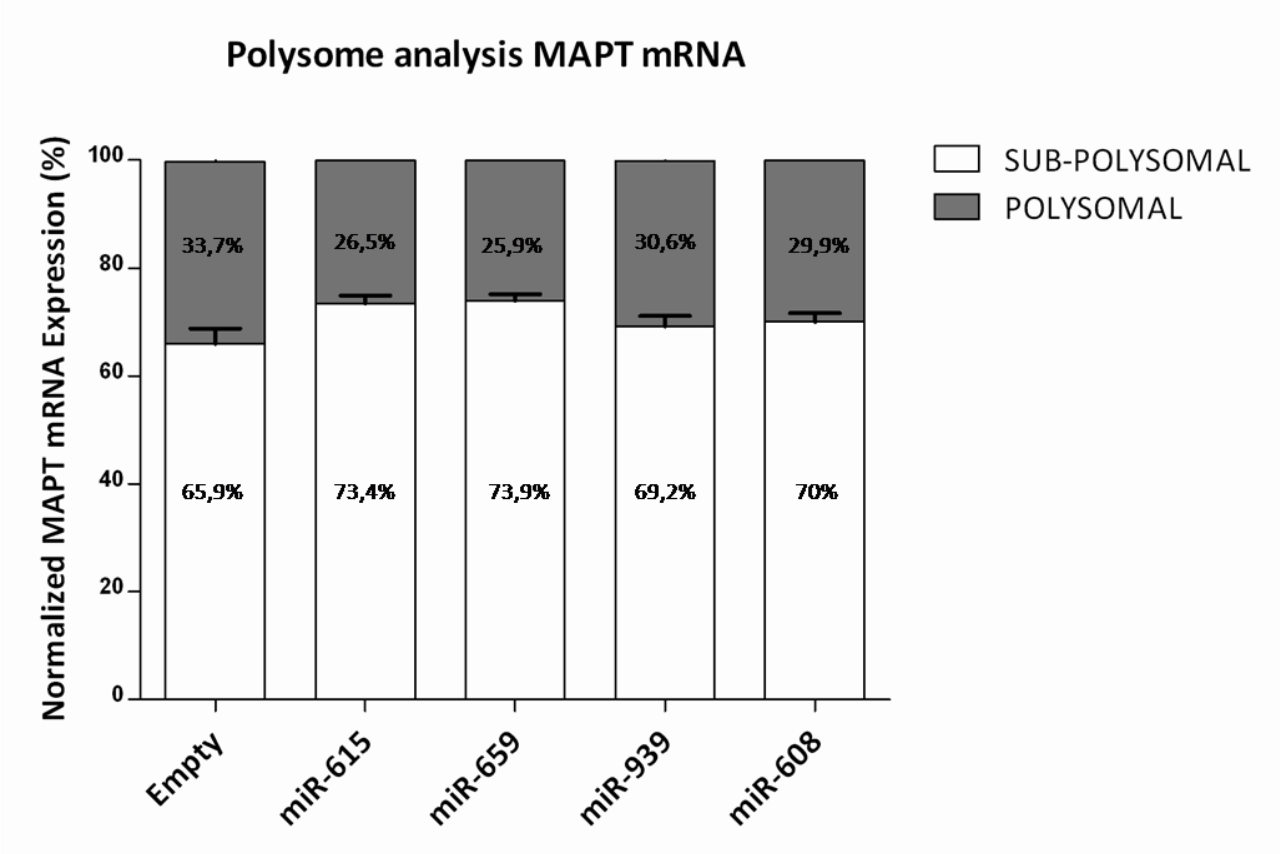


Figure 5.2.7Effect of miRNAs over-expression on MAPT mRNAs in polysomal and sub-polysomal compartments. KELLY cell line was transfected with the over-expressing plasmid for all the selected miRNAs and the empty control. After 48h the mRNA-ribosomal complexes were separated through sucrose gradient centrifugation and RNA was extracted from the sub-polysomal and polysomal compartments. Real Time PCR was performed for the quantification of MAPT mRNAs in the sub-polysomal and polysomal fractions. MAPT mRNAs expression was normalized against the housekeeping HPRTI transcript. Percentages of MAPT transcripts measured in the two fractions were calculated on the sum of $2^{-\Delta Ct}$ values of MAPT mRNAs from polysomal and sub-polysomal compartments. Data derived from three biological experiments. (Mean ± SEM).*

*This work was done in collaboration with Dr. Paola Zuccotti from laboratory of Translational Genomics in CIBIO.

CHAPTER SIX: DISCUSSION AND FUTURE PROSPECTIVES

6.1 PROGRANULIN AND HYPOXIA

Previous study by our collaborators identified an up-regulation of GRN mRNA and progranulin protein levels in SK-N-BE cell line exposed to chronic hypoxia (Piscopo *et al*. 2010). The same hypoxic samples with the relative controls were analyzed during our collaboration, for the expression of miR-659-3p, showing a significant negative correlation between miR-659-3p and GRN mRNA expression. This opposite trend suggested a possible link of regulation. Indeed miR-659-3p was found to bind GRN 3'UTR as shown by luciferase assays performed at 24h and 48h. Moreover we showed that miR-659-3p over-expression down-regulates endogenous progranulin expression, as reported by ELISA assays and western blot analysis in HeLa cell line. Rademarker and colleagues demonstrated that a common genetic variant localized on GRN 3'UTR (rs5848), located at the predicted binding site for miR-659-3p, represents a risk factor for FTLD-TDP (Rademakers *et al*. 2008). They reported that in presence of the T variant allele there was a stronger binding of miR-659-3p, resulting in a major reduction of progranulin. Our results are additionally showing that miR-659-3p could bind GRN 3'UTR even in presence of the wild type C-allele, regulating progranulin expression. Interestingly the sequencing of GRN 3'UTR performed in HeLa and SK-N-BE identified the presence of the variant T-allele in SK-N-BE cell line. Indeed SK-N-BE cell line expresses an heterozygous TC genotype, with a major prevalence of T-allele, while HeLa cell line carries the homozygous CC genotype. Following the pull-down experiments, GRN capture samples showed an high enrichment of miR-659-3p in SK-N-BE cell line. In order to investigate whether the putative correlation in oxidative stress condition takes place also in vivo, RNA extracted from a rat model of global perinatal asphyxia was used for the quantification of miR-659-3p. This model mimics an acute event of intra-uterine asphyxia around the time of birth (Bjelke *et al*. 1991). Neural and behavioral alterations have been identified in this model (Boksa *et al*. 1995; Chen *et al*. 1997a; Chen *et al*. 1997b; Dell'Anna *et al*. 1997) and a delayed cell death in frontal cortex, striatum and cerebellum was reported after eight days from the asphyxia induction (Dell'Anna *et al*. 1997; Van de Berg *et al*. 2002). Perinatal asphyxia is a major cause of brain damage in the newborn and could be responsible for different delayed neurologic disorders (Calamandrei *et al*. 2004).

Therefore the correlation between progranulin and miR-659-3p was investigated in vivo in a different scenario of oxidative stress, in which free radical generation blocks the anti-oxidant defenses, playing a role in the neurodegeneration of different adult and infant brain diseases (Calamandrei *et al.* 2004). After 20 minutes of global intrauterine asphyxia there was an up-regulation of GRN mRNA, that was statistically significant at pnd1 and pnd4, with a major increase at pnd4 compared to controls. Interestingly samples in which progranulin was significantly up-regulated (pnd1 and pnd4), showed a reduction of miR-659-3p expression, that was significant at pnd4. Thus post natal day 4 demonstrated a significant negative correlation between miR-659-3p and GRN mRNA expression in a rat model of perinatal asphyxia.

Some studies reported that chronic hypoxia condition can repress the level of proteins involved in miRNA biogenesis, such as Drosha and Dicer (Caruso *et al*. 2010; Ho *et al.* 2012). This finding could suggest that the down-regulation trend of miR-659-3p observed in hypoxia samples might be caused by a global reduction of protein involved in miRNAs production. However, in our analysis on SK-N-BE cell line under chronic hypoxia condition we screened additional miRNAs that did not show the same down-regulation pattern. These miRNAs were putative candidates for the regulation of GRN mRNA, but they reported an unaltered or up-regulated expression after hypoxia (data not shown). They were excluded from the study since their expression was not correlated with progranulin trend in chronic hypoxia experiments performed in neuroblastoma cell line and in perinatal asphyxia samples. Thus we can exclude the possibility that miR-659-3p expression could be influenced and down-regulated by Dicer reduction in hypoxic condition, even though Dicer and Drosha were not analyzed and investigated in this study.

Progranulin has a role as inflammation antagonist (Tang *et al.* 2011) and this function, in the attenuation of the inflammatory response, seems to be important for the increase of neurons survival following CNS injury, caused by oxidative stress (Martens *et al*. 2012). Effectively up-regulation of progranulin expression induced by oxidative insults, activates survival pathways that lead to the rescue of cortical neurons from cell death (Xu *et al.* 2011). For this reason progranulin is considered a neuroprotection factor and its functional brain reduction causes a decreased neuronal protection with reduction of survival signaling, as observed in FTD patients (Martens *et al*. 2012; Xu *et al*. 2011). In this work we reported that miR-659-3p and progranulin expression are reverse regulated in hypoxic condition.

Interestingly it was demonstrated that hypoxia induces the production of several miRNAs implicated in the modulation of important cellular responses involving apoptosis and inflammation (Taganov *et al*. 2006; Zhang *et al*. 2012). It would be interesting to investigate if miR-659-3p could represent a negative regulator of this neuroprotection pathway and which are the additional miRNAs that instead lead to the up-regulation of progranulin and induction of survival responses. Several hypoxamirs promote HIF expression and activity, or represent transcriptional targets of HIF (Loscalzo *et al*. 2010; Semenza *et al*. 2012; Greer *et al.* 2012; Kelly *et al*. 2011). It would be of great use to analyze if miR-659-3p down-regulation is linked with HIF hypoxic adaptation activity or with HIF-independent pathways that involved additional proteins, such as p53, mTOR or the endoplasmic reticulum stress related proteins. These findings will show the regulation pathways that should be activated in case of oxidative stress, in order to have progranulin neuroprotection function and preserve the neuronal activity. The regulative factors for the up-regulation of progranulin would be particularly important for the development of therapeutic strategies for the potential cure of neurodegenerations such as FTD. Further studies on this direction would also reveal important information for the potential cure of perinatal asphyxia. Indeed exist a narrow period of time between the insult and the development of CNS injury, during which therapeutic strategies could be effective (Lorek *et al*. 1994; Northington *et al*. 2001). Maybe during this period the neuroprotection role of progranulin could reduce brain damage or the risk of cognitive and attentional disorders during the development.

6.2 IDENTIFICATION OF miRNAs INVOLVED IN THE POST-TRANSCRIPTIONAL REGULATION OF GRN EXPRESSION

In the last period accumulated evidences showed that complexity of higher eukaryotes is a highly regulated process which correlates with an increase in the size of non-coding regions (Levine *et al*. 2003; Barrett *et al*. 2012). The untranslated regions and introns are the main regions involved in this highly regulated process that control gene expression. 3'UTR regions regulate gene expression at multiple levels, such as the 3'end formation and polyadenylation or they influence mRNA stability, subcellular localization and translation efficiency (Conne *et al.* 2000, Mignone *et al*. 2002, Chabanon *et al.* 2004). The 3'UTR functions as binding region for numerous regulatory proteins and miRNAs to complete its regulative role (Barrett *et al*. 2012).

In the present study, post-trascriptional regulation of different miRNAs acting on GRN 3'UTR was investigated using several techniques. A recent method, named miR-CATCH, developed by the laboratory of Catherine Greene and her co-workers, was applied for the analysis of miRNAs that can physiologically bind to GRN mRNA in different cell lines. This approach was used to complement the initial bioinformatic prediction performed to identify the putative miRNAs binding on GRN. The predictive outcome was the result of three computational programs, in order to increase the specificity of the prediction. However, it is estimated that bioinformatics softwares have a false-positive rate of 24-70% (Sethupathy *et al.* 2006) and different algorithms generate often heterogeneous lists that are barely overlapped. On the contrary miR-CATCH method has the advantage to identify miRNAs that could physiologically bind to the entire full length mRNA, including the 5'UTR and the coding regions. Indeed the isolation of an endogenous mRNA allows the identification of all the innate miRNAs and Argonaute proteins bind to its native conformation, reflecting a natural biological process (Hassan *et al*. 2013). The method was applied to different cell lines in order to compare the regulation played by our putative miRNAs of interest on GRN mRNA in different cell environment. Following the miR-CATCH experiments for the capture of GRN mRNA, only miR-659-3p showed a substantial and significant level of enrichment for all the cell lines analyzed, even though the fold enrichments were not similar. The difference could be caused by the heterogeneous level of GRN mRNA enrichment obtained with miR-CATCH

strategy, applied in the different cell lysate. This difference could probably reflect a varied endogenous quantity of GRN mRNA naturally present in the analyzed neuroblastoma cell lines. A disadvantage of miR-CATCH method was the possibility to isolate only miRNAs that are express at the endogenous level, for this reason miR-608 and miR-615-5p, that are absent or express in a minimal and hardly detectable quantity, were not found in the elute samples. Further improvements in the miR-CATCH protocol, should possibly focus on analyzing transfected cells. This will allow the isolation of targets or miRNAs expressed in lower levels at basal condition. The indicated modification could be of great help for a major use of the technique to different purposes.

The activity of a miRNA in a particular cell type could be influenced by the target competition process (Hassan *et al.* 2013). This could in part explain why miR-939-5p was absent from the pull-down samples, although this miRNA was detectable in the analyzed cell lines. Although the bioinformatic screening appeared to be complex, the use of different softwares is fundamental for the subsequent analysis of miRNA recognition elements on GRN 3'UTR. MiR-939-5p was strongly predicted to target GRN mRNA, showing three putative binding sites located on GRN 3'UTR with good level of free binding energy (ΔG). For this reason miR-939-5p was used to analyze the effect of its over-expression on GRN mRNA and progranulin, even though miR-939-5p was not found enriched in the capture samples. Similarly, the strong bindings predicted in silico for miR-608, miR-615-5p and miR-659-3p led to investigate their role on GRN mRNA and progranulin protein expression. Luciferase assays performed with GRN 3'UTR reporter plasmids, showed down-regulation of luciferase activity, upon the over-expression of the miRNAs of interest at 24h and 48h. The reduction of luciferase was significant upon the over-expression of miR-659-3p. Even though different level of luciferase down-regulation was obtained according to the different part of GRN 3'UTR used for the assay, possibly indicating that the sequence and the length of the portion of GRN 3'UTR used, could influence the recognition process. To this matter it is reported that RNA regulatory elements function in a context of specific secondary structure (Chen *et al.* 2006). Therefore the differentiated pattern of luciferase reduction obtained could be attributed to the difference in secondary structures elicited by the different 3'UTR portions, containing the same miRNAs binding sites. Indeed the binding of a specific miRNA could be promoted and enhanced by a particular secondary structure. In case of the entire GRN 3'UTR, miR-659-3p showed the best luciferase reduction at 24h and 48h. However at 48h

there was a considerable decrease of luciferase, upon the over-expression of miR-939-5p and miR-615-5p. The effect of miRNAs over-expression on the endogenous progranulin level was analyzed in KELLY cell line using western blot. All the over-expressed miRNAs caused a decrease of progranulin protein compared to control. In this experiment the major effect was observed with miR-939-5p and miR-659-3p. These findings demonstrated the role of the selected miRNAs on the regulation of GRN mRNA, showing a reduction in the translation of progranulin. Since in HeLa cell line miR-939-5p appeared to be expressed in a considerable level at physiological conditions, a miR-inhibitor was used to block the endogenous level of miR-939-5p. The block of miR-939-5p induced an up-regulation of progranulin expression, likely indicating a possible role of miR-939-5p in the control of progranulin expression.

During the regulation of the target mRNA, miRNAs can have two main functions: degradation of the target mRNA, in case of a perfect complementarity binding and translational repression in the presence of an imperfect binding interaction (Krol *et al*. 2010). In order to investigate the effect of putative miRNAs of interest on GRN, Real Time PCR reactions were performed to quantify the GRN mRNA level after the over-expression of the miRNAs of interest. However the over-expression of only miR-615-5p induced a significant down-regulation of GRN mRNA compared to control. These findings led us to the use of polysomes analysis, in order to verify whether the putative miRNAs of interest could regulate the level of progranulin by affecting the translation efficiency of GRN mRNA. Following the transfection of the selected miRNAs, there was an increase in the amount of GRN mRNA measured on the sub-polysomal compartments, indicating a translation repression of GRN mRNA induced by miRNAs. Reduction of translation efficiency of GRN mRNA was more prominent after the over-expression of miR-939-5p, miR-615-5p and miR-659-3p. Therefore these data suggested that the selected miRNAs can regulate progranulin expression through a mechanism of translation repression. In these cell conditions the stronger effect of down-regulation was observed with miR-659-3p, miR-939-5p and miR-615-5p.

6.3 IDENTIFICATION OF miRNAs INVOLVED IN THE POST-TRANSCRIPTIONAL REGULATION OF MAPT EXPRESSION

Mir-CATCH method was applied for the isolation of all the different five isoforms of MAPT, obtaining good levels of fold enrichments in KELLY and SK-N-BE cell lines and a weak enrichment in SH-SY5Y cell line. However, the profiling of selected miRNAs showed high miR-659-3p and miR-939-5p enrichments in all the eluted samples compared to controls, indicating a possible mechanism of regulation. The bioinformatics prediction derived from three different softwares identified one particular binding site for miR-608 located on Fragment V of MAPT 3'UTR region. Luciferase assays were subsequently performed with the over-expression of miR-659-3p and miR-608. The logic behind choosing miR-608 and miR-659-3p was due to the fact that miR-939-5p was also predicted to bind the coding region of MAPT mRNA, while miR-CATCH strategy results and bioinformatic predictions showed miR-608 and miR-659-3p as good putative candidates for the regulation of MAPT 3'UTR. The over-expression of these miRNAs showed a significant luciferase down-regulation using the full length MAPT 3'UTR and the specific fragment V containing the putative binding sites. These results demonstrated an effective binding of miR-659-3p and miR-608 on MAPT 3'UTR region. Initial bioinformatics prediction analysis derived from two softwares had previously identified putative binding sites for miR-615-5p and miR-939-5p on MAPT mRNAs. For this reason, in the following experiments, the usual panel of selected miRNAs (miR-659-3p, miR-608, miR-939-5p and miR-615-5p) was applied to investigate their role on the regulation of MAPT mRNAs and tau expression. Western blot experiment reported a down-regulation of tau protein upon the over-expression of miR-659-3p and miR-608, whereas miR-615-5p and miR-939-5p caused a tiny increase of tau expression. The function of miRNAs regulation on MAPT mRNA isoforms was analyzed with Real Time PCR in order to quantify the levels of MAPT mRNAs after over-expression of selected miRNAs. MAPT mRNAs levels were not down-regulated after miRNAs over-expression, indicating that miR-659-3p and miR-608 that affect the protein production, do not induce MAPT mRNAs degradation. Polysomal analysis was applied in order to understand the mechanism played by miR-659-3p and miR-608 on the down-regulation of tau. The quantity of MAPT mRNAs found on the sub-polysomal compartments increased upon the over-expression of all the selected

miRNAs. The major shift of MAPT mRNAs from the polysomal to the sub-polysomal compartment was observed with the over-expression of miR-659, miR-608 and miR-615. However, the small increase of tau expression obtained with miR-615 and miR-939 does not correlate with the translational repression observed upon their over-expression. Since the over-expression induced by these miRNAs do not down-regulate tau protein expression, the translational repression of MAPT mRNAs could be caused by an indirect effect. Additional mechanisms could have a role in the regulation of MAPT mRNAs and tau expression played by miR-615 and miR-939. Further experiments and additional validations for these miRNAs would probably clarify their role on MAPT mRNAs regulation. However, the data indicated that miR-659 and miR-608 are potential regulators of tau expression, targeting MAPT 3'UTR and reducing the translational efficiency of MAPT mRNAs.

6.4 FUTURE PERSPECTIVES

This study provides direct evidence and validation of the role of some miRNAs in the modulation of GRN and MAPT mRNAs and protein expression. However, additional analysis of miRNAs enriched in the capture samples derived from miR-CATCH experiments would reveal other potential regulators of GRN and MAPT mRNAs. With the use of miRCURY LNA Real Time PCR plates for miRNAs expression profiling in capture samples compared to scrambled controls, we would like to identify other miRNAs involved in GRN and MAPT regulation.

Moreover, in order to correlate the regulation played by the selected miRNAs on GRN and MAPT, we plan to investigate the expression of these putative miRNAs on samples derived from post-mortem brains of FTD patients. Since only 20% of all FTLD-TDP cases have loss of function mutations in GRN (Baker *et al*. 2006; Cruts *et al*. 2006) and remaining patients present the same pathological manifestations often with a family history, other genetic factors could be involved in the regulation of progranulin level (Nicholson *et al*. 2011). One genetic factor was identified by Rademakers and co-workers in a study in which they demonstrated that a common genetic variant localized on GRN 3'UTR (rs5848) is a risk factor for FTLD-TDP (Rademakers *et al*. 2008). SNP rs5848 is located at the predicted miR-659-3p binding site and, in presence of the T variant allele, there was a stronger miR-659-3p binding, resulting in a major reduction of progranulin. In agreement with these findings,

reduced progranulin levels were found in brain lysates of FTLD patients carrying two copies of rs5848 risk T-allele, compared to the levels observed in patients carrying two copies of the rs5848 C-allele (Rademakers *et al*. 2008). An additional study showed that rs5848 polymorphism can significantly influence serum progranulin level, demonstrating lower level of serum progranulin in rs5848 TT carriers compared to the level measured in serum of CT and CC carriers (Hsiung *et al*. 2011). However another article reported no association of rs5848 with FTLD in three European cohorts (Rollinson et al. 2011). In the present study we suggested that additional common genetic variabilities observed on miRNAs binding sites found on GRN and MAPT 3'UTRs could alter the normal regulation of GRN and MAPT mRNAs, representing potential risk factors for FTD generation. In the SNPs analysis performed for GRN, six of the sixteen SNPs found on GRN 3'UTR region were located on putative miRNAs binding sites. According to the method developed by Saba and colleagues in 2014 (Saba *et al*. 2014), two SNPs identified on GRN 3'UTR caused an enhanced miRNA binding: one is located on the first miR-608 binding site, while the other one is the SNP rs5848 found by Rademarkers and coworkers. Except for one SNP that showed no effect, all the other 3 SNPs induced a decrease in miRNAs binding. Interestingly the miR-608 binding region located on Fragment V of MAPT 3'UTR could be affected by one SNP and two different events of small insertion or deletion (indel). Seven of the different variations identified on the Fragment V of MAPT 3'UTR showed an alteration of the free binding energy for the predicted miRNAs interactions. In particular four variations caused an increased miRNAs binding: one is located in miR-659-3p binding site, two are present in the second miR-608 binding site and the last is localized on the third miR-608 binding site. Whereas all the other variations caused a reduction in the predicted miRNAs binding to Fragment V of MAPT 3'UTR. It would be fascinating to analyze if these SNPs could be associated with FTD and represent possible risk factors for the development of neurodegeneration. Neurological diseases are indeed strongly influenced by natural genetic variability that exists in the population (Hulse and Cai, 2013; Lukiw, 2013). In this scenario, it should be considered not only the target region but also the miRNA gene variability. Evidence has shown that polymorphisms found on miRNA genes could interfere with their function, resulting in phenotypic variation and disease susceptibility (Zorc *et al*. 2012). A single variation may be enough to alter miRNA-mediate regulation and might induce altered fine tuning gene expression with unpredicted consequences.

Genetic association studies are necessary in both cases, in which the genetic variability affects miRNAs target regions or miRNAs encoding regions. These type of analysis would reveal if the variability can contribute to a disease state, playing a role as a risk factor in sporadic patients or modifying the pathological process in patients having causal mutations.

Therefore further analysis related to the investigation of genetic factors involved in progranulin and tau regulation would be important for a better understanding of the pathology of FTD and other neurodegenerative disorders.

In the present research project, the effect of two miRNAs suggests a possible common mechanism of regulation for progranulin and tau. Indeed the expression of miR-659-3p and miR-608 induces a down-regulation of progranulin and tau production, blocking the translational expression of GRN and MAPT mRNAs. These genes cause frontotemporal dementia through different mutations and trigger events. In addition the causal mutations of GRN and MAPT lead to opposite effects, showing reduced progranulin levels but increased tau expression in FTD patients. Possible common pathways of regulation would be important for the development of similar therapeutical strategies. Although the therapeutical approaches should be conducted in two different directions.

The research of common regulations or pathways for progranulin and tau could potentially lead to the identification of additional key factors that are deregulated in all types of FTD pathology. These factors would be extremely important for the development of future therapeutical strategies and biomarkers for the early diagnosis of the disease state.

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List of my contributions:

- ❖ All the experiments performed and the results reported in the content are obtained with independent research, except the parts indicated by an asterisk.

- ❖ My contribution to the work "**A network of RNA and protein interactions in Fronto Temporal Dementia"** published in **Frontiers in Molecular Neuroscience** in March 2015:

- Paragraphs related to the genes: *FUS, GRN, CHMP2B, C9ORF72.*
- Contribution to the introduction and discussion parts.
- Production of the following figures: miRNAs biogenesis (Figure 1), schematic representation of the functional domains in FUS/TLS (Figure 3 A), schematic representation of the progranulin structure (Figure 4 B), schematic representation of CHMP2B and overview of the genomic structure of the *C9ORF72* gene (Figure 5 A and C).
- Production of the following tables: Table of protein localization of different genes associated with FTD (Table 1), Table related to the list of mutations in *FUS* (Table 3), Table related to the list of mutations in *GRN* (Table 5), Table related to the list of mutations in *CHMP2B* (Table 7), Table related to the list of mutation in *C9ORF72* (Table 8).

- ❖ My contribution to the chapter "**Circulating cell-free microRNAs as biomarker for neurodegenerative diseases**", accepted for publication in the book "**Mapping Nervous System Diseases via MicroRNAs**".

  Production of the following paragraphs:

  2. Biomarkers in Neurodegenerative diseases

  3. miRNA biogenesis and function

  4. Circulating miRNAs

  4.1 Alzheimer's disease and circulating miRNAs

  4.2 Parkinson's disease and circulating miRNAs

Production of:

- Table 1 Alzheimer's Disease and circulating miRNAs
- Table 2 Parkinson's Disease and circulating miRNAs
- Table 3 Amyotrophic Lateral Sclerosis and circulating miRNAs

❖ My contribution to the work "**Reduced miR-659-3p levels correlate with progranulin increase in hypoxic conditions: implications for frontotemporal dementia." (manuscript attached):**

- Bioinformatic prediction of miR-659
- Cloning of GRN 3'UTR reporter plasmid (pmiRGLO-GRN3'UTR)
- Cloning of miR-659 over-expressing plasmid (pSiUx-miR-659)
- Transfection of miR-659-3p, miR-181a-5p and Empty vector in HeLa cell line
- Real Time PCR for the validation of miR-659-3p and miR-181a-5p over-expression at 24h and 48h
- Luciferase experiments at 24h and 48h
- Protein analysis of endogenous progranulin after the over-expression of miR-659-3p and miR-181a-5p through ELISA assays and western blot
- Design of the GRN capture oligonucleotide and pull down assay of GRN mRNA in SK-N-BE cell line
- Real Time PCR for GRN mRNA enrichment in capture samples compared to scrambled controls
- Real Time PCR for miR-659-3p enrichment in capture samples compared to scrambled controls
- Real Time PCR for the detection of miR-659-3p in all the hypoxic SK-N-BE samples compared to normoxic controls
- Real Time PCR for the detection of miR-659-3p in all the asphyctic newborn rats samples compared to relative controls

Results produced from the manuscript:

- **Figure 2**
- **Figure 3**
- **Figure 4**

- **Figure 5**
- Real Time data performed for all the hypoxic SK-N-BE samples and relative controls used for the statistical analysis reported in the **Figure 7**
- Real Time data performed for all the samples reported in the **Figure 8 B**

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REVIEW
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A network of RNA and protein interactions in Fronto Temporal Dementia

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Frontotemporal dementia (FTD) is a neurodegenerative disorder characterized by degeneration of the fronto temporal lobes and abnormal protein inclusions. It exhibits a broad clinicopathological spectrum and has been linked to mutations in seven different genes. We will provide a picture, which connects the products of these genes, albeit diverse in nature and function, in a network. Despite the paucity of information available for some of these genes, we believe that RNA processing and post-transcriptional regulation of gene expression might constitute a common theme in the network. Recent studies have unraveled the role of mutations affecting the functions of RNA binding proteins and regulation of microRNAs. This review will combine all the recent findings on genes involved in the pathogenesis of FTD, highlighting the importance of a common network of interactions in order to study and decipher the heterogeneous clinical manifestations associated with FTD. This approach could be helpful for the research of potential therapeutic strategies.

Keywords: FTD, TDP-43, FUS, progranulin, tau, CHMP2B. C9ORF72

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Frontotemporal Dementia

Despite 90% of the human genome being transcribed to RNA, only 1.2% of genomic sequence is protein-coding, indicating that a huge proportion of non-coding RNAs (ncRNAs) are likely to participate in a number of physiological processes in cell types, including neurons (Lander et al., 2001; Birney et al., 2007; Wilhelm et al., 2008; Clark et al., 2011). The transcribed precursors of messenger RNAs (pre-mRNA) undergo splicing, such that the non-coding introns are removed and exons are combined variably to produce an RNA that would code for protein (Pandit et al., 2008). The pre-mRNAs undergoes alternative splicing producing mature messenger RNAs (mRNAs) which are then expressed in specific tissues and cell types in different stages of development. These mRNAs then associate with the ribosomal machinery to be translated into proteins in the cytoplasm. Non-coding RNAs (among which microRNAs and long non-coding RNAs), might regulate the translation of specific mRNAs, thereby representing a post-transcriptional mechanism exerting a fine-tuned control in the production of specific proteins.

microRNAs (miRNAs) are a group of small non-coding RNAs of 21–22 nt with important regulatory roles on the post-transcriptional expression of target mRNAs (Bartel, 2009; Ghildiyal and Zamore, 2009). MiRNAs are generating from longer transcripts of different lengths called primary transcripts (pri-miRNAs), usually transcribed by RNA polymerase II, from intragenic or intergenic DNA regions (Lee et al., 2004; Garzon et al., 2010). The pri-miRNAs are processed in the nucleus by the micro-processor complex, formed by an RNase III enzyme, Drosha, and its cofactor DiGeorge

syndrome critical region in gene eight termed (DGCR8) (Lee et al., 2003). The process lead to the production of small hairpin structure of 70–100 nt called precursor miRNAs (pre-miRNAs). Pre-miRNAs are exported to the cytoplasm through Exportin 5 (Kim, 2004), where they are further processed by an RNase III nuclease, Dicer to produce RNA duplex (Bernstein et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001). One strand is loaded on the RNA-Induced Silencing Complex (RISC) and associated with Argonaute-2 (Ago2) to interact with the target mRNA. The miRNA-RISC complex induces mRNA downregulation through two different ways: mRNA cleavage in case of perfect complementarity between miRNA and target mRNA or translation inhibition if there is an imperfect binding (Wahid et al., 2010) (**Figure 1**). In case of perfect complementarity, Ago2 is the protein involved in the cleavage of the target mRNA in humans (Liu et al., 2004). However, in animals, translational repression is the most frequent way of action for miRNAs (Huntzinger and Izaurralde, 2011; Pasquinelli, 2012), although the exact process is still unknown since is not clear if the repression occur at the initiation step or during the translation process (Wahid et al., 2010). Even the mechanisms for target regulation played by miRNAs are still unclear, the target mRNA could be repressed by the promotion of deadenylation, sequestration of miRNAs and target by stress granules and P-Bodies (Valencia-Sanchez et al., 2006), disruption of translation initiation or protein degradation caused by RISC after translation (Tang et al., 2008).

The enormous content of non-coding RNA (ncRNA) in the cell intrigues its role and function in the cells. LncRNAs are defined as transcripts longer than 200 nucleotides and lacking an appreciable open reading frame (usually less than 100 amino acids). They may be transcribed by RNA polymerase II (RNA Pol II) or RNA Pol III, and may undergo splicing or comprise of a single exon. In contrast to small ncRNAs, lncRNAs tend to be poorly conserved evolutionarily and regulate gene expression by diverse mechanisms that are not entirely understood. As a functionally diverse macromolecule, the biological roles of lncRNAs cannot be determined solely from their nucleotide sequence, secondary structures, or genomic locations (Ng et al., 2013).

Recent work has begun to elucidate the roles of some lncRNAs, such as architectural function in nuclear paraspeckles (Sunwoo et al., 2009; Souquere et al., 2010), transcriptional co-regulators (Feng et al., 2006; Bond et al., 2009), and as endogenous competing RNAs (ceRNAs) (Cesana et al., 2011; Tay et al., 2011). LncRNA expression is abundant in cells of the CNS (Mehler and Mattick, 2007; Mercer et al., 2008) and recent studies have suggested that lncRNAs play crucial roles in spatial-temporal control of gene expression in brain development (Mercer et al., 2008). They have also known to be involved in brain development, neural differentiation and maintenance, synaptic plasticity, cognitive function and memory, and in aging and neurodegenerative disorders (Wu et al., 2013b).

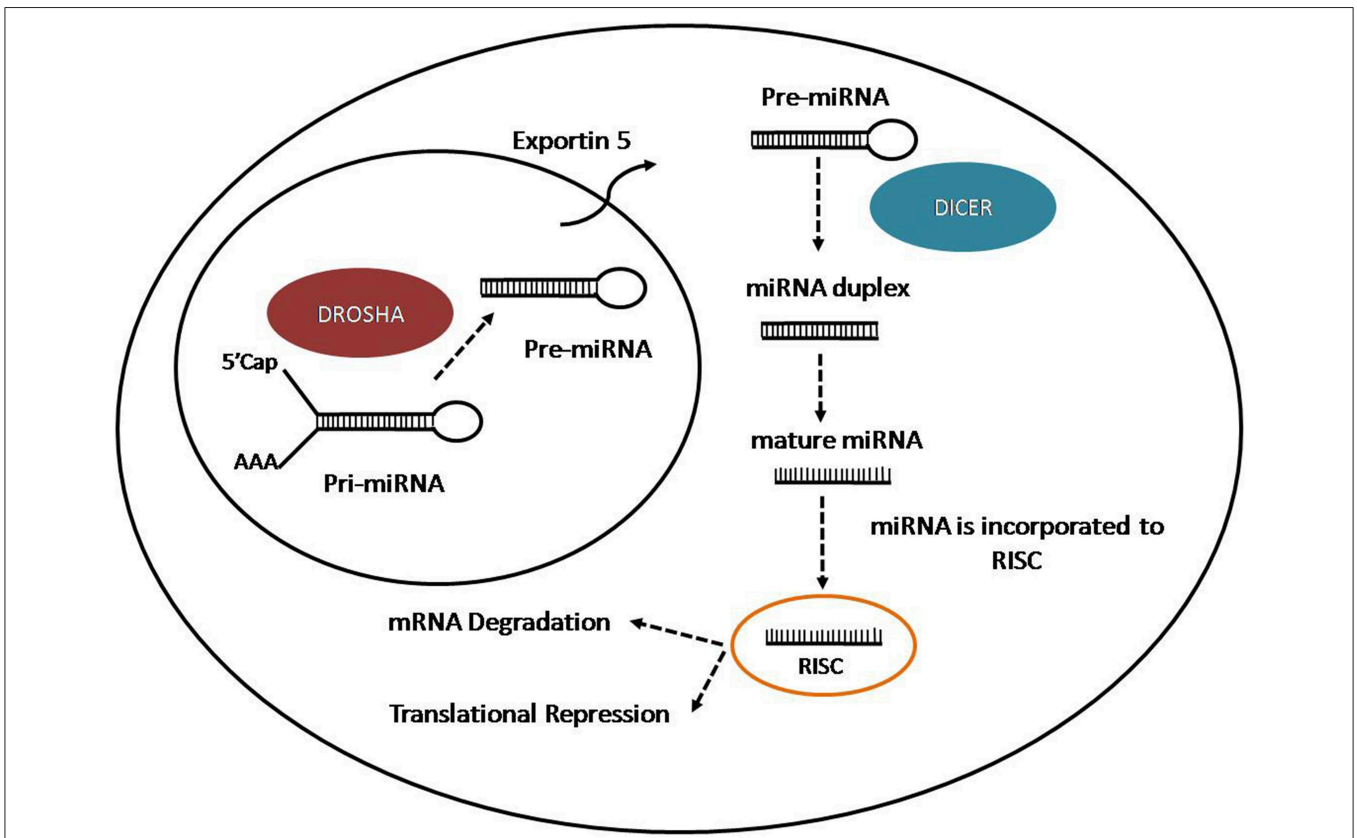

FIGURE 1 | Representation of miRNA biogenesis.

Though different mechanisms may play a role in causing neurodegenerative disorders, recent studies show increasing evidence of abnormalties in RNA processes, highlighting the possible putative role of RNA in neurodegeneration. An mRNA gain-of-toxic-function has been proposed for some neurodegenerative diseases (Osborne and Thornton, 2006; O'Rourke and Swanson, 2009; Todd and Paulson, 2010) whereas other neurodegenerative disorders are driven through altered or lost non-coding RNA, RNA splicing and RNA binding activities (Gallo et al., 2005; Cooper et al., 2009; Lagier-Tourenne et al., 2010).

Fronto temporal lobar degeneration (FTLD) is the most common cause of dementia after Alzheimer's disease. The clinicopathological spectrum of FTLD includes frontal and temporal variants of frontotemporal dementia (FTD), primary progressive aphasia, semantic dementia, Cortico-basal degeneration (CBD), progressive supranuclear palsy (PSP), progressive subcortical gliosis (PSG) and FTD with motor neuron disease (FTD–MND) (Bugiani, 2007). Moreover, despite Amyotrophic Lateral Sclerosis (ALS) and FTD being two different neurodegenerative disorders, they often share genetic, neuropathological and clinical characteristics; therefore they are considered part of the same spectrum of diseases (Ling et al., 2013). Frontotemporal dementia symptoms can also be present along with disabling muscle weakness and osteolytic bone lesions, in IBMPFD1 (Inclusion body myopathy with early-onset Paget disease with or without Frontotemporal dementia 1).

It is estimated that one in seven people in the US might develop a neurodegenerative disorder in their lifetime, with dementia being one of the leading causes of death in US (Thies and Bleiler, 2011). Though this broad spectrum of disorders has been studied based on protein aggregation and research has been focusing on protein functions and alterations, emerging avenues in research unravels the role of RNA and RNA processing in contributing to neurodegeneration (Belzil et al., 2013).

To date, FTD has been linked to mutations in seven different genes (*TARDBP*, *FUS*, *MAPT*, *GRN, VCP*, *CHMP2B*, *C9ORF72*).

Findings that showed the presence of ubiquitinated protein TDP-43 in sporadic cases of ALS with FTD further linked these two diseases (Arai et al., 2006; Neumann et al., 2006). Following these findings, mutations in the gene coding for the RNA binding protein TDP-43 were discovered in ALS cases (Kabashi et al., 2008; Sreedharan et al., 2008; Van Deerlin et al., 2008) and FTD cases (Borroni et al., 2009; Kovacs et al., 2009).

With the broadening knowledge on the impact of impaired RNA binding proteins in mediating the disease process, mutations in the fused in sarcoma/translocated in liposarcoma (*FUS/TLS*) gene were found to account for an additional 5% of familial ALS and few rare cases of FTD (Kwiatkowski et al., 2009; Vance et al., 2009).

TDP-43 and FUS share similar structural and functional properties with a likely role in multiple steps of RNA processing and they are both linked to RNA metabolism. The pathological accumulation of these proteins is observed in over 90% ALS and 50% FTD patients. These studies also highlight that errors in RNA processing might be enough to initiate the disease process.

MAPT mutations were observed in several FTD families with abnormally phosphorylated tau proteins being isolated from neuroectoderm cells of patients. Mutations present in the C terminal repeat domains lead to the inhability of abnormal tau protein to bind microtubules, thus leading to its instability and accumulation and causing neuronal degeneration (Bugiani, 2007). FTD with tau inclusions was characterized as a tauopathy and dubbed FTLD-tau.

However, a different class of patients were found to have had accumulated ubiquitin and ubiquitin-associated proteins (FTLD-U). Co-localization of abnormal proteins with ubiquitin in the nucleus and perikaryon of neuronal cells, indicated the involvement of proteasome dysfunction in the pathology. Analysis of significant genes on chromosome 17, close to the MAPT locus, led to the discovery of mutations in *GRN* (Baker et al., 2006; Cruts et al., 2006). GRN is known to be involved in the cell cycle control and motility.

Studies on an ALS/FTD-affected Scandinavian family (Morita et al., 2006) and on IBMPFD1 families suggested the possible role of mutations in chromosome 9 in FTD. The disorder was associated to mutations in *VCP,* encoding the valosin-containing protein essential for ubiquitin-mediated protein degradation (Watts et al., 2004; Johnson et al., 2010).

Other FTLD mutations are located on chromosome 3 (FTD-3), in the *CHMP2B* gene, which encodes for a protein involved in degradation of surface receptor proteins and formation of endocytic multivesicular bodies (Skibinski et al., 2005).

Another link between ALS and FTD are the large intronic hexanucleotide repeat expansions in the *C9ORF72* gene located on chromosome 9 found in ALS, FTD, or ALS/FTD cases (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Gijselinck et al., 2012).

This review will focus on the single genes known to have implications in FTD and their altered functions in the diseased state. The ultimate aim is to explorepossible functional connections between these seven diverse genes and describe a network in which a possible common thread might be represented through RNA mediated processes.

TARDBP (TDP 43)

Human TDP-43 was discovered in 1995 in a screen for transcriptional repressors of the trans-active response (TAR) DNA binding element of the HIV-1 virus, and thus the gene is named TARDNA Binding Protein (*TARDBP*) (Ou et al., 1995). *TARDBP* is composed of six exons and maps on chromosome 1p36.22.

The protein *TARDBP* produces is being labeled as TDP-43 due to its molecular weight of 43 KDa (Neumann et al., 2006). *TARDBP* is ubiquitously expressed in various human tissues (**Table 1**) including brain and spinal cord (Wang et al., 2008a). To date, 34 different TDP-43 mutations have been discovered in 131 different FTD and ALS families (Cruts et al., 2012). Pathogenic mutations observed in TDP-43 are highlighted in **Table 2**.

Structure

TDP-43 is a 414 amino acids protein (**Figure 2A**) containing two RNA recognition motifs (RRMs), a glycine-rich low sequence

TABLE 1 | Protein localisation of different genes associated to FTD.

Gene	Genomic location	Protein	Tissue localization in the brain	Cell type	Subcellular localization
TARDBP	Chromosome 1 p36.22	TDP-43	Cerebral cortex, hippocampus, lateral ventricle, cerebellum and spinal cord	Endothelial, neuronal, glial cells, neuropil and cell in granular and molecular layer, Purkinje cells	Nucleus and cytoplasm
FUS	Chromosome 16 p11.2	FUS	Cerebral cortex, hippocampus, lateral ventricle and cerebellum	Endothelial, neuronal, glial cells, neuropil and cell in granular and molecular layer, Purkinje cells	Nucleus and cytoplasm
MAPT	Chromosome 17 q21.3	Tau	Cerebral cortex, hippocampus, lateral ventricle and cerebellum	Neuronal, glial cells, neuropil and cell in granular and molecular layer, Purkinje cells	Cytoskeleton, cytoplasm, nucleus and plasma membrane
GRN	Chromosome 17 q21.31	Progranulin	Cerebral cortex, hippocampus, lateral ventricle and cerebellum	Neuronal, glial, endothelial cells and cell in granular layer	Vesicles, endoplasmic reticulum, golgi, extracellular space
VCP	Chromosome 9 p13.3	VCP	Cerebral cortex, hippocampus, lateral ventricle and cerebellum	Endothelial, neuronal, neuropil, glial cells and cell in granular and molecular layer, Purkinje cells	Endoplasmic reticulum, nucleus, cytoplasm
CHMP2B	Chromosome 3 p11.2	CHMP2B	Lateral ventricle	Neuronal cells	Cytosol, endosome, nucleus, mitochondria
C9ORF72	Chromosome 9 p21.2	C9ORF72	Cerebral cortex, hippocampus and lateral ventricle	Endothelial, neuropil, glial cells	Cytoplasm, nucleus, cytoskeleton

The information provided are derived through integration of two different databases (http://www.genecards.org/ and http://www.proteinatlas.org/) and literature reported in the text.

TABLE 2 | List of mutations in *TARDBP* and their characteristic phenotypes.

Subtypes of Dementia	Mutation	Change in amino acid	Type	References
FTD	g.6142C>T	p.N12	Pathogenic	Luquin et al., 2009
FTD	g.9253C>T	A90V	Pathogenic	Sreedharan et al., 2008
FTD/PSP	g.14575A>G	K263E	Pathogenic	Kovacs et al., 2009
ALS/FTD	g.14588A>G	N267S	Pathogenic	Corrado et al., 2009
FTD	g.14671G>A	G295S	Pathogenic	Benajiba et al., 2009
FTD	g.14932G>A	A382T	Pathogenic	Chiò et al., 2010

FTD, Frontotemporal Dementia; PSP, Progressive supranuclear palsy.
All the information reported in the table is derived from a cumulative study of the literature and the database: http://www.molgen.ua.ac.be/ADMutations/default.cfm?MT=0&ML=2&Page=FTD.

complexity prion-like domain (Wang et al., 2013). A nuclear localization signal motif (NLS) and a nuclear export signal motif (NES) allow TDP-43 to shuttle between the nucleus and the cytosol (Buratti and Baralle, 2001).

Localization and Function

Though TDP-43 expression is seen in the nucleus with low cytosolic localization (Ayala et al., 2005), there is a significant cytosolic TDP-43 expression especially in large motor neurons where TDP-43 has an additional role in mRNA transport as a neuronal activity responsive factor in dendrites thus promoting dendritic branching (Wang et al., 2008a; Barmada et al., 2010; Kabashi et al., 2010).

TDP-43 was found to be accumulated in cytoplasmic stress granules due to oxidative stress (Colombrita et al., 2009). Stress granules are aggregations, formed after cell insults such as oxidative stress or heat shock that temporarily store non-translating mRNAs, small ribosome subunits, RNA-binding proteins and translation initiation factors (Buchan and Parker, 2009). Formation of stress granules protects the cells, allowing a translational block and initiation of repair processes (Anderson and Kedersha, 2008).

Upregulation of nuclear TDP-43 has also been shown to provide protection to primary neurons against glutamate induced excitotoxicity (Zheng et al., 2012). These findings also suggest that TDP-43 regulates synaptic plasticity by governing the transport and splicing of synaptic mRNAs. In a recent review, Belzil and co-authors postulate that altered TDP-43 could lead to impaired hippocampal plasticity and render neurons more vulnerable to cellular stressors (Belzil et al., 2013).

TDP-43 is highly conserved from human to *C. elegans*, both in the RNA binding motifs and in the carboxy-terminal portion (Ayala et al., 2005). *In situ* hybridization studies showed that TDP-43 is expressed very early in the brain and spinal cord of zebrafish (Shankaran et al., 2008) suggesting that it plays an important role in nervous system development.

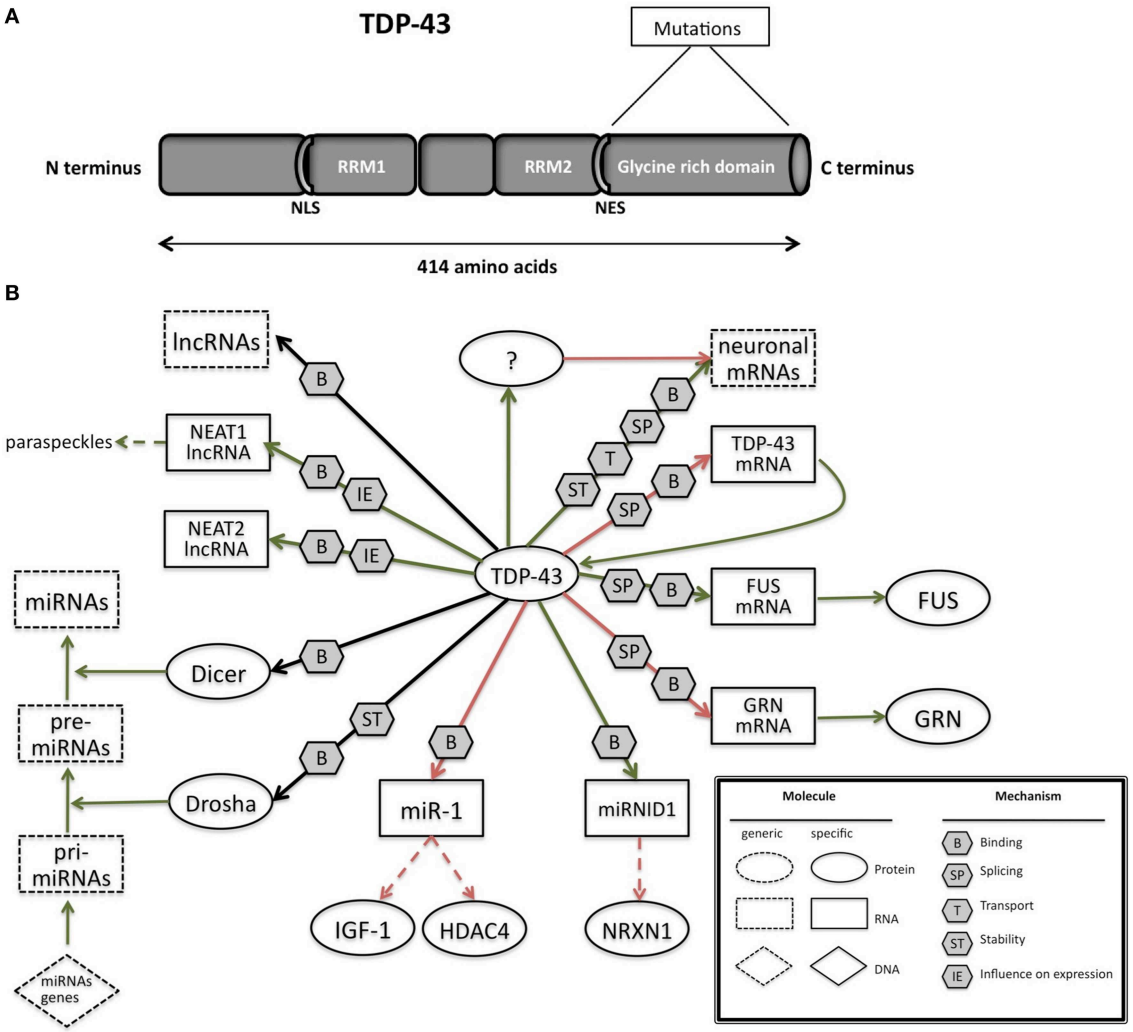

FIGURE 2 | (A) TAR DNA-binding protein 43 (TDP-43) contains two RNA-recognition motifs (RRM1 and RRM2), a carboxy-terminal glycine-rich domain, a bipartite nuclear localization signal (NLS), and a nuclear export signal (NES). Mutations are mainly located in the glycine-rich domain. **(B)** The network of interactions of TDP-43 with proteins and RNAs. Green arrows indicate binding interactions or processes that result in activation or increased expression. Red arrows indicate binding interactions or processes that result in inhibition of activity or reduced expression. Black arrows indicate binding interactions or processes whose result can be either positive or negative. Dashed arrows indicated indirect processes. Symbols as in Legend. lncRNAs, long non-coding RNAs; IGF-1, insulin-like growth factor 1; HDAC4, histone deacetylase 4; NRXN1, neurexin 1; TDP-43, TAR DNA binding protein; FUS, fused in sarcoma; GRN, progranulin; NEAT1, nuclear-enriched autosomal transcript 1; NEAT2, nuclear-enriched autosomal transcript 1.

Implications of RNA in Pathogenesis

Many studies have linked TDP-43 to neurodegenerative disorders, including ALS and FTLD (Neumann et al., 2006; Lagier-Tourenne et al., 2010; Lee et al., 2012). Janssens and Van Broeckhoven (2013) have highlighted the increasing evidence of role of impaired RNA metabolism in TDP-43-driven neurodegeneration.

TARDBP primary transcript undergoes alternative splicing to produce eleven different mRNAs including the one encoding TDP-43. Seven of these are shorter transcripts which are generated through the seven different splicing reactions within exon 6 of *TARDBP* pre-mRNA using a combination of four different 5′ donor sites and four different 3′ acceptor sites (Wang et al., 2004a).

In few ALS cases a smaller TDP-43 isoform (∼28 KDa) was observed additionally to the 43 kDa isoform, lacking exon 3 and a significant portion of exon 6-encoded amino acids (Strong et al., 2007). This smaller isoform lacks the carboxy-terminal portion of the protein and is thought to be associated with disease pathology (Neumann et al., 2006).

Converging lines of evidence in research suggest that TDP-43 regulates RNA in various ways (**Figure 2B**; Lee et al., 2012). The RRM1 domain of TDP-43 is critical for its binding to single-stranded RNA (Ou et al., 1995; Buratti and Baralle, 2001; Wang

et al., 2004a; Ayala et al., 2005). TDP-43 preferentially binds UG repeats, but is also found to be associated with non-UG repeat sequences (Buratti and Baralle, 2001; Ayala et al., 2005; Polymenidou et al., 2011; Tollervey et al., 2011).

Pathological TDP-43 aggregates are ubiquitinated and phosphorylated. Under normal conditions, these forms are not readily detectable in brain tissues, thus making them disease-specific. Over-expression of full-length TDP-43 in a variety of transgenic animal models lead to the presence of phosphorylated TDP-43 aggregates similar to ALS and FTD cases (Wegorzewska et al., 2009; Shan et al., 2010; Stallings et al., 2010; Wils et al., 2010; Xu et al., 2010a). The phosphorylated form has a longer half-life than the non-phosphorylated form thus leading to accumulation of phosphorylated proteins. Despite the progress toward describing the full spectrum of TDP-43 pathology in human neurodegenerative diseases, the fundamental question of whether TDP-43 dysfunction mediates neuro-degeneration through gain of toxic function or a loss of normal function remains unanswered (Lee et al., 2012).

Upon depletion of TDP-43 from adult mouse brain with antisense oligonucleotides, levels of 601 mRNAs, including FUS, GRN and other transcripts involved in neurodegeneration, were altered, along with 965 varied splicing events. RNAs depleted by the reduction of TDP-43 were coded by genes with long introns (Polymenidou et al., 2011).

In-vivo searches for TDP-43 RNA targets in mouse (Polymenidou et al., 2011), human brain (Tollervey et al., 2011), rat cortical neurons (Sephton et al., 2011), a mouse NSC-34 cell line (Colombrita et al., 2012), and a human neuroblastoma cell line (Xiao et al., 2011) revealed that there are more than 6000 RNA targets which constitutes to about 30% of total transcriptome. TDP-43 was found to preferentially bind to introns (including deep intronic sites), 3′ untranslated regions (UTRs), and non-coding RNAs (Polymenidou et al., 2011; Tollervey et al., 2011), indicating a multifaceted role in RNA maturation. TDP-43 can influence splice site selection by binding to exon-intron junctions and intronic regions, mRNA stability and transport by binding on 3′UTRs. A substantial amount of mRNAs regulated by TDP-43 at splicing levels were involved in neuronal development or in neurological diseases (Tollervey et al., 2011). Additional data show that when TDP-43 is reduced the levels of several other mRNAs increase. As the affected mRNAs include more than 300 mRNAs without TDP-43 binding sites, these observation point toward an indirect mechanism (Polymenidou et al., 2011) of modulation.

i-CLIP experiments have also shown that TDP-43 binds to long ncRNAs (lncRNAs), including nuclear-enriched autosomal transcript 1 (NEAT1) and metastasis-associated lung adenocarcinoma transcript 1 (MALAT1, also called NEAT 2) (Tollervey et al., 2011). Expression of both lncRNAs is elevated in FTD patients with TDP-43 inclusions, thus correlating with their increased association with TDP-43 (Tollervey et al., 2011).

The binding of TDP-43 to small (<200 base) ncRNAs and miRNAs remains largely unexplored. However, the association of TDP-43 with Drosha microprocessor (Ling et al., 2010) and Dicer complexes (Freibaum et al., 2011; Kawahara and Mieda-Sato, 2012) provides a suggestive role of TDP-43 involvement in miRNA biogenesis. Indeed, let-7b miRNA is downregulated, whereas miR-663 is upregulated upon reduction of TDP-43 (Buratti et al., 2010). Di Carlo and collegues demonstrated that TDP-43 directly interacts with Drosha and controls its stability at different levels. Moreover, TDP-43 is also involved in the Drosha substrate recognition as in the regulation mediated by Drosha of Neurogenin 2, an important and master gene in neurogenesis (Di Carlo et al., 2013).

Fan et al. (2014) have performed CLIP-seq analysis to examine the small RNAs (pri-miRNAs, miRNAs, and piRNAs) bound to TDP-43 and found that a novel miRNA (miR-NID1), which is processed from the intron five of human neurexin 1 gene (*NRXN1*), interacts with TDP-43 and represses expression of *NRXN1*. Neurexins are cellular proteins that function as cell adhesion molecules and receptors in the vertebrate nervous system, involved in synaptic development including calcium signaling, heterogeneous cell-to-cell adhesion and synaptogenesis (Craig et al., 2006; Bottos et al., 2011) Disruptions or mutations of *NRXN1* have been reported to associate with autistic spectrum disorder (ASD), mental retardation, and schizophrenia (Reichelt et al., 2012).

Recent studies by King and colleagues identified a physical interaction between TDP-43 and miR-1 family which is known to be involved in smooth muscle gene repression in heart and an opposing myogenic differentiation (King et al., 2014). TDP-43 overexpression in skeletal muscle led to decrease of miR-1 and increased protein levels of the miR-1 family targets, IGF-1 and HDAC4. These results demonstrate that TDP-43 could influence miRNA regulation through a physical interaction by limiting their bioavailability for RISC loading and offer a mechanism by which mature miRNAs can be differentially regulated.

The expression of TDP-43 is tightly autoregulated through a complex interplay between transcription, splicing, and 3′ end processing (Avendaño-Vázquez et al., 2012): TDP-43 overexpression in humans and mice leads to activation of a 3′ UTR intron which results in excision of proximal polyA site (PAS) which in turn activates a cryptic PAS and prevents TDP-43 expression through a nuclear retention mechanism.

The above mentioned studies have highlighted that TDP-43 is linked to various mRNAs and non-coding RNAs, in a neuronal context wherein it mediates effects through splicing or interaction with Drosha and Dicer complexes. It is also involved in its autoregulation mediated at the RNA level.

Additionally, TDP 43 is known to interact with MATR3, a DNA RNA binding protein. Their interaction was confirmed to be RNA based. Mutations in this gene have been linked to cases of ALS. The authors further report that the phenotype observed in patients with MATR3 was a combination of those observed in cases of ALS and myopathy. Clinical symptoms were similar to patients with VCP mutations (Johnson et al., 2014).

FUS

FUS, (fused in sarcoma, also called TLS: translocated in liposarcoma) belongs to the TET family of RNA binding proteins involved in many different cellular processes (Bertolotti et al., 1996; Law et al., 2006; Tan and Manley, 2009). *FUS,* located

on chromosome 16 at locus p11.2, encodes a multifunctional protein able to bind and interact with single stranded RNA and double stranded DNA, participating in different aspects of RNA metabolism (Shelkovnikova et al., 2014).

Structure

FUS is characterized by different domains (**Figure 3A**): a N-terminal domain with transcriptional activating properties mainly composed of glutamine, glycine, serine, and tyrosine residues (Law et al., 2006), a glycine rich region, a RNA binding domain, and a highly conserved C-terminus capable of binding DNA, RNA and splicing factors (Law et al., 2006).

Localization and Function

FUS is mainly localized in the nucleus (Colombrita et al., 2009; Van Blitterswijk and Landers, 2010; Kawahara and Mieda-Sato, 2012) but it is also actively implicated in other cellular processes that occur in the cytoplasm such as mRNA transport, mRNA stability and translation (Buratti and Baralle, 2010; Colombrita et al., 2011). Indeed FUS was reported to shuttle between the nucleus and the cytoplasm, exporting to the cytoplasm spliced mRNAs in ribonucleoprotein complexes (Zinszner et al., 1997). Particularly, upon stimulation in hippocampal neurons FUS was reported to accumulate in the spines of mature dendrites, where local translation occurred (Fujii and Takumi, 2005). FUS immunoreactivity was also observed in dendritic spines in mature primary cultures and in adult hippocampus *in situ* (Belly et al., 2005; **Table 1**).

The C-terminal part of FUS encodes for a non-classic nuclear localization signal (**Figure 3A**; Iko et al., 2004) that is necessary for nuclear import, as it was demonstrated through the generation of deletion mutant lacking 13 amino acids in the C-terminal part of FUS(Dormann et al., 2010).

Several papers reported that mutations and aberrations of *FUS* are linked to the pathogenesis of frontotemporal degeneration (FTD) as well as familial and sporadic ALS (Kwiatkowski et al., 2009; Vance et al., 2009), as reported in **Table 3**. Moreover, FUS accumulates in inclusions in the cytoplasm of autopsied spinal cords and brains of sporadic and familial ALS and FTD. FUS inclusions are not only observed in presence of *FUS* mutations, as they were found in patients with different or unknown genetic defects such as sporadic ALS, ALS/dementia or FTLD (with or without progranulin mutations), FUS or TDP43 mutation-linked familial ALS, SOD1-negative familial ALS. These inclusions were also positive for TDP43/ubiquitin and p62 (Deng et al., 2010).

ALS/FTD patients show mutations mainly in the Glycine rich region and C-terminal part (Lagier-Tourenne et al., 2010). The mechanism underlying the pathogenesis of FUS mutations was related to FUS nucleus/cytoplasmic imbalance since ALS mutations increase its localization in the cytoplasm, observed through immunostaining of FUS in postmortem ALS brain samples (Kwiatkowski et al., 2009), or through the analysis in neuroblastoma cell lines of the subcellular localization of recombinant mutant FUS fused either to green fluorescent protein (GFP) (Kwiatkowski et al., 2009; Morlando et al., 2012), an N-terminal hemagglutinin (HA) tag (Vance et al., 2009), a C-terminal V5-His tag, or an N-terminal myc tag in HeLa (Ito et al., 2011).

Both the loss of FUS nuclear function and the potential gain of toxic effect by FUS in the cytoplasm could explain pathogenesis (Shelkovnikova et al., 2014).

Very few studies so far reported FTD cases associated with *FUS* mutations. The first analysis of *FUS* in FTD patients showed a novel missense mutation in the glycine-rich region of FUS, predicted to be pathogenic by *in silico* analysis (Van Langenhove et al., 2010). Subsequently another study found novel missense mutations in patients with familial ALS with features of frontotemporal dementia (FALS/FTD) and one with familial ALS with parkinsonism and dementia (FALS/PD/DE) (Yan et al., 2010). Recently, another study found two novel heterozygous missense mutations in *FUS* in patients with behavioral variant FTD (bvFTD), however the pathogenicity of these mutations needs to be further investigated in other screening (Huey et al., 2012).

FUS has been reported to co-localize with TDP-43 in nuclear complexes (Kim et al., 2010b; Ling et al., 2010) and in larger cytoplasmic complexes (Kim et al., 2010b). Purified FUS has also been reported to interact with purified His-tagged TDP-43 *in vitro* in an RNA-independent manner, associated to the C-terminal region of TDP-43 (Kim et al., 2010b). These ubiquitously expressed binding proteins seem to have similar and complementary functions.

Only the mutant form of FUS was found in stress granules in reponse to translational arrest (Bosco et al., 2010). FUS and TDP-43 were observed to co-localize in cytoplasmic aggregations of ALS/FTLD-affected neurons (Da Cruz and Cleveland, 2011). Dormann and colleagues found stress granule markers such as PABP-1 and eIF4G co-deposited with FUS inclusions in sections of post-mortem brain and spinal cord tissue from familial ALS-FUS and sporadic FTDLD-U. On the contrary, TDP inclusions did not show any co-localization with stress granules proteins in HeLa transiently transfected with the mutated form of FUS, after heat shock for 1 h (Dormann et al., 2010). Another study reported that ubiquitin-positive inclusions in frozen post-mortem tissue from FTLD-TDP patients were not stained with anti-FUS antibodies (Neumann et al., 2009b), therefore FUS and TDP-43 are not always found in the same inclusions or aggregates.

The relation between FUS and TDP-43 is reported as a delicate equilibrium, where small alteration on their relative quantity and presence in nucleus/cytoplasm could very likely cause serious problem over a long period (Colombrita et al., 2012), which might be an accumulation of events due to an alteration of their targetome.

Implications of RNA in Pathogenesis

FUS is involved in pre-mRNA splicing (**Figure 3B**), by interacting with splicing factors such as SRm160, PTB, and serine/arginine rich proteins (SR proteins) (Yang et al., 1998; Meissner et al., 2003). In addition the recent sequencing approaches applied to clarify the function and identify the targets of FUS reinforced its fundamental role in splicing (Colombrita et al., 2012) by revealing its binding to intronic sequences or to splice site acceptors.

Similarly to many other splicing factors, FUS can bind the C-terminal domain of RNA polymerase II and prevent

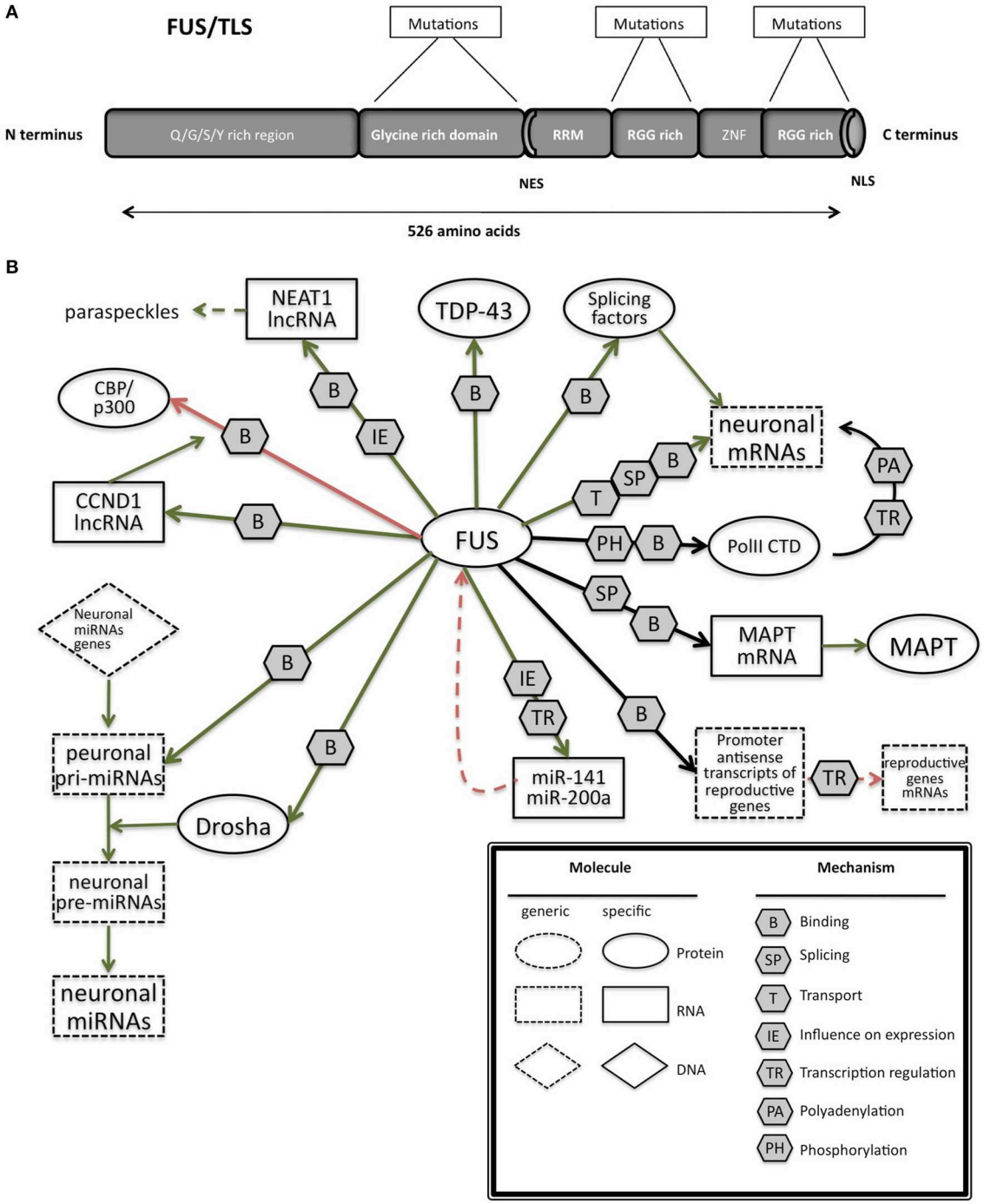

FIGURE 3 | (A) Schematic representation of the functional domains in FUS/TLS. FUS contains a N-terminal part enriched in glutamine, glycine, serine and tyrosine residues (QGSY region), a glycine-rich region, a nuclear export signal (NES), an RNA recognition motif (RRM), repeats of arginine, glycine, glycine (RGG), a zinc finger motif (ZNF), and a C-terminal nuclear localization signal (NLS). Most of the mutation are localized in the glycine rich region and in the last 17 amino acids of the NLS part. **(B)** The network of interactions of FUS with proteins and RNAs. Green arrows indicate binding interactions or processes that result in activation or increased expression. Red arrows indicate binding interactions or processes that result in inhibition of activity or reduced expression. Black arrows indicate binding interactions or processes whose result can be either positive or negative. Dashed arrows indicated indirect processes. Symbols as in Legend. lncRNAs, long non-coding RNAs; IGF-1, insulin-like growth factor 1; HDAC4, histone deacetylase 4; NRXN1, neurexin 1; TDP-43, TAR DNA binding protein; FUS, fused in sarcoma; MAPT, microtubule-associated protein tau; NEAT1, nuclear-enriched autosomal transcript 1; CCND1, G1/S-specific cyclin-D1; CBP, CREB-binding protein; p300, Histone acetyltransferase p300; PolII CTD, Carboxy-terminal Domain of the RNA polymerase II.

TABLE 3 | List of mutations in *FUS* and their characteristic phenotypes.

Subtypes of Dementia	Mutation	Change in amino acid	Type	References
FTD	g.4961A>G	M254V	Unclear	Van Langenhove et al., 2010
bvFTD	g.31183985C>T	P106L	Unclear	Huey et al., 2012
ALS/FTD	g.31185031G>A	G206S	Unclear	Yan et al., 2010
FALS/PD/DE	g.31191418C>T	R521C	Unclear	Yan et al., 2010
ALS/FTD	g.31191419G>A	R521H	Pathogenic	Broustal et al., 2010

ALS, Amyotrophic lateral sclerosis; FTD, Frontotemporal Dementia; bv FTD, behavioral variant Frontotemporal Dementia; FALS, Familial Amyotrophic lateral sclerosis; PD, Parkinson's disease; DE, Dementia.

All the information reported in the table is derived from a cumulative study of the literature and the database: http://www.molgen.ua.ac.be/ADMutations/default.cfm?MT=0&ML=2&Page=FTD.

the premature hyperphosphorylation of Ser2 in the C-terminal domain of RNA polymerase II. Moreover the lack of FUS leads to an accumulation of RNA polymerase II at the transcription start site with a shift toward abundance of mRNA isoforms with early polyadenylation (Schwartz et al., 2012).

FUS can bind to the promoter antisense strand transcript of some genes such as Ptprn2, Xrn1, Gak, or Glt1d1 and this interaction downregulates the transcription of the coding sense strand, but this effect seems to be specific for some genes enriched with GO terms connected to the reproductive process(Ishigaki et al., 2012).

As FUS was shown to regulate RNA polymerase II at many more gene promoters than the genes reported for splicing defects, its role on transcription could be a separated function in addition to the regulation on splicing (Schwartz et al., 2012). However, a small proportion of FUS target regions is localized in exonic sequences and in the 3′UTRs (Hoell et al., 2011), suggesting another potential role, such as the transport of mRNAs or the control of mRNA stability and translation (Fujii et al., 2005; Fujii and Takumi, 2005). A model was suggested, in which FUS is released from actin filaments, when cytoskeletal organization collapses, becoming free to be linked to the mRNA that is transported to the local translational machinery in the spines (Fujii and Takumi, 2005).

Recent techniques, like HITS-CLIP or RIP-CHIP were also used to identify FUS binding motif, but all the studies lead to the common assumption that FUS binds to specific secondary structures on its RNA targets and a primary sequences analysis is not sufficient (Colombrita et al., 2012; Ishigaki et al., 2012).

Interestingly, silencing of *FUS* was reported to alter splicing events in genes, such as *MAPT,* that have an important neuronal function (Ishigaki et al., 2012). This finding leads an unexpected connection between these two genes, both involved in the pathogenesis of FTD. In particular, FUS was shown to help the skipping of *MAPT* exon 10 in primary cortical neurons (Ishigaki et al., 2012). The alternative splicing of *MAPT* exon 10 is known to have a causative role in FTD as discussed later (*MAPT* paragraph).

FUS is also involved in microRNA biogenesis (Morlando et al., 2012), specifically interacting with pri-miRNAs and Drosha, and helping the recruitment of Drosha for the correct miRNA processing in neuronal cells. Several miRNAs like miR-9, miR-132, and miR-125b whose biogenesis is controlled by FUSare important for neuronal functions, neuronal differentiation, and synaptogenesis (Morlando et al., 2012). Additionally miR-9 and miR-132 have also been shown to control neurite extension and branching through downregulation of Foxp2 (Forkhead box protein P2) (Clovis et al., 2012) Moreover this role of FUS seems to be prominent in neuronal cells compared to non-neuronal cells, such as HeLa cells, in which the proportion of miRNAs affected by *FUS* knockdown was lower. Indeed the mutations known to induce a cytoplasmic delocalization of FUS would impede its nuclear role as pri-miRNA processor. Though the balance of nuclear and cytoplasmic FUS seems necessary, the sole role of nuclear FUS should not be neglected and further investigations would be needed to clarify its biological function within this cell compartment. Recently, the same laboratory demonstrated the presence of a regulatory loop in which FUS can enhance the expression of miR-141 and miR-200a, which in turn regulate FUS, through a binding on its 3′UTR. This pathway seems to be affected in the presence of one mutation found in two ALS patients (Dini Modigliani et al., 2014).

FUS is also reported to bind lncRNAs. The binding to lncRNA CCND1 induces an allosteric change in FUS, thus in turn permits its interaction with CBP/p300. As FUS represses CBP/p300-mediated transcription by inhibiting their histone acetyltransferase (HAT) functions (Wang et al., 2008a), in the presence of ncRNA CCDN1, CBP/p300-mediated transcription is repressed.

The nuclear-enriched abundant transcript 1 (NEAT1) produces two types of lncRNAs from the same promoter NEAT1_1 and NEAT1_2 (Nishimoto et al., 2013). FUS was shown to bind NEAT1_2, known to assemble and organize the core proteins of paraspeckles (Wang et al., 2008a; Hoell et al., 2011; Lagier-Tourenne et al., 2012), which represent a storage for the rapid release of RNAs during stress condition or a nuclear retention of long hyperedited transcripts (Prasanth et al., 2005; Chen and Carmichael, 2009). According to observations and data obtained from cultured cells, transgenic mice and human postmortem tissue, paraspeckles represents an important protective cell mechanism during stress conditions (Nakagawa et al., 2011; Nakagawa and Hirose, 2012; Shelkovnikova et al., 2014).

Paraspeckels are present in almost all the cultured cells (Fox and Lamond, 2010), but in normal tissues are found only in cells that contain high levels of NEAT1_2 RNA and coherently, in neurons where NEAT1 is express at low levels, paraspeckles are not observed (Nakagawa et al., 2011).

The presence of FUS in paraspeckles was confirmed in different cell lines by three studies (Naganuma et al., 2012; Nishimoto et al., 2013; Shelkovnikova et al., 2014) Moreover, NEAT1 was shown through PAR-CLIP to be a target of both WT and mutant FUS (Hoell et al., 2011).

Paraspeckles are found in spinal motoneurons of patients at early stage of ALS. The possibility that aging induces an increase in the level of NEAT1_2 was ruled out due to the fact that human control cases were older that ALS cases of an average of 10 years. However, the process that induces an up-regulation of NEAT1_2 lncRNA during the early phases of ALS is still unknown (Nishimoto et al., 2013). Overall FUS seems to play a key role on the regulation of RNA at different levels, acting on transcription, splicing, transport, and stability of mRNA with a particular function in microRNA biogenesis and interaction with non-coding RNAs.

MAPT (Tau)

MAPT (microtubule associated protein) encodes for protein Tau and is located on chromosome 17q21.3. The gene, which is 150 kb-long, contains 16 exons, out of which 11 are expressed in CNS (Wolfe, 2012).

Structure

The protein consists of a projection domain, including an acidic and a proline-rich region, which interacts with cytoskeletal elements (**Figure 4A**). The N-terminal part is involved in signal transduction pathways by interacting with proteins such as PLC-γ and Src-kinases. The C-terminal part, referred to as the microtubule binding domain, regulates the rate of microtubules polymerization and is involved in binding with functional proteins such as protein phosphatase 2A (PP2A) or presenilin 1 (PS1) (Luna-Muñoz et al., 2013).

Localization and Function

Tau is a microtubule-associated protein which is found in abundance in the axons of Central nervous system (CNS) and Peripheral nervous system (PNS) (Binder et al., 1985; Couchie et al., 1992; **Table 1**). It is also observed in astrocytes and oligodendrocytes in the CNS. The tau pre-mRNA undergoes alternative splicing at exons 2, 3, and 10 to give six different possible isoforms. Inclusion of exon 10 generates 4-repeat or 4R tau, while exclusion forms 3-repeat or 3R tau. In neurons this ratio is controlled throughout development, emphasizing the importance of this balance for neuronal functions.

Implications of RNA in Pathogenesis

In FTD populations, *MAPT* mutation frequency ranges from 8 to 50%. To date, 44 different *MAPT* mutations, either mis-sense or splice mutations or both, have been discovered in 138 different families (Cruts et al., 2012). The list of pathogenic mutations observed in *MAPT* are reported in **Table 4**). Most missense mutations alter ability of tau to bind to microtubules, thus leading to the formation of inclusion in neurons and glia, called neurofibrillary tangles (NFT) (Lee et al., 2005).

About half of the mutations in *MAPT*, however, are associated with alteration of splicing of exon 10 and increase the ratio of 4R to 3R. The mutations near exon 10 5′splice site enhance inclusion of exon 10 either by altering the linear *cis*-splicing elements or by

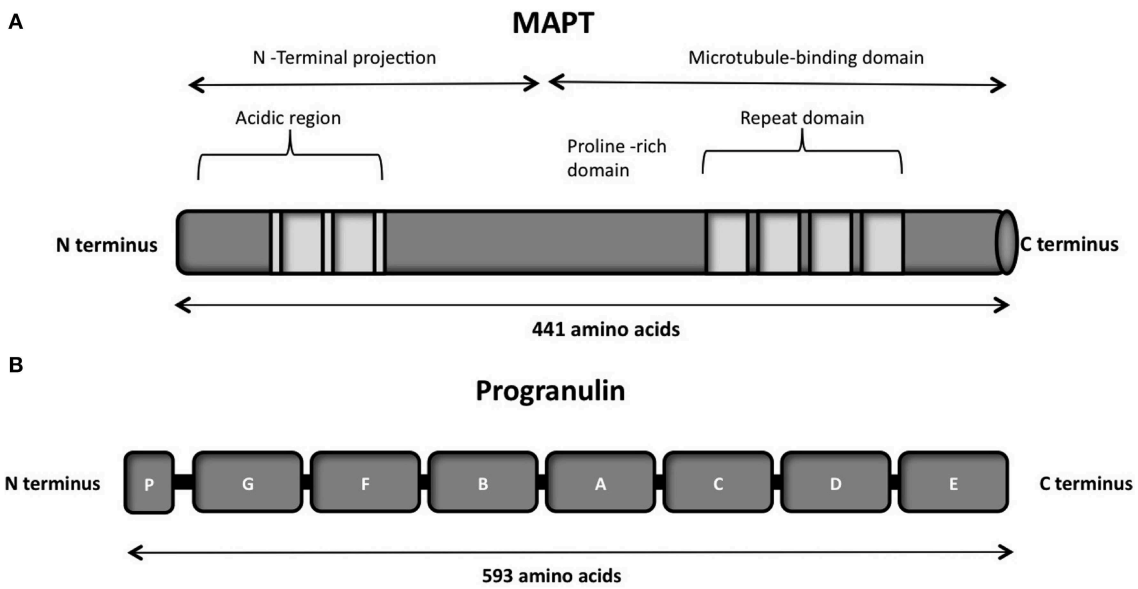

FIGURE 4 | (A) Schematic representation of the functional domains of the largest tau isoform (441 amino acids 2N4R isoform). The N-terminal projection domain, including an acidic and a proline-rich region, interacts with cytoskeletal elements. The N-terminal part is also involved in signal transduction pathways by interacting with proteins such as PLC-γ and Src-kinases. The C-terminal part, also known as the microtubule-binding domain, regulates the rate of microtubules polymerization and is involved in binding with proteins such as protein phosphatase 2A (PP2A) or presenilin 1(PS1). **(B)** Schematic representation of the progranulin structure encoded by the major human transcript containing all the 13 exons. The lettered boxes on the progranulin scheme represent the individual granulin domains containing cystein-rich motifs.

TABLE 4 | List of mutations in *MAPT* and their characteristic phenotypes.

Subtypes of dementia	Mutation	Change in amino acid	Type	References
FTD	g.75756G>A	R5H	Pathogenic	Hayashi et al., 2002
FTD	g.110018A>C	K592T	Pathogenic	Rizzini et al., 2000
FTD	g.110026A>G	I595V	Pathogenic	Grover et al., 2003
FTD	g.110044C>G	L601V	Pathogenic	Kobayashi et al., 2003
FTD	g.110063G>T	G607V	Pathogenic	Schenk, 1959
FTD	g.110065G>A	G608R	Pathogenic	Van der Zee et al., 2006
FTD	g.123725T>G	N614K	Pathogenic	Wszolek et al., 1992
FTD	g.123729_123731delAAG	DeltaK616 (alias ΔK280; ΔK281)	Unclear	Rizzu et al., 1999
FTD	g.123740T>C	L619	Pathogenic	D'Souza et al., 1999
FTD	g.123774A>C	N631H	Pathogenic	Iseki et al., 2001
FTD	g.123776T>C	N631	Pathogenic	Brown et al., 1996
FTD	g.123789C>A	P636T	Pathogenic	Lladó et al., 2007
FTD	g.123789C>T	P636S	Pathogenic	Bugiani et al., 1999
FTD	g.123790C>T	P636L	Pathogenic	Hutton et al., 1998
FTD	g.123802G>A	S640N	Pathogenic	Iijima et al., 1999
FTD	g.123802G>T	S640I	Pathogenic	Kovacs et al., 2008
FTD	g.123803T>C	S640	Pathogenic	Spillantini and Goedert, 2000
FTD/PSP	g.123806G>A	Intronic	Pathogenic	Spillantini et al., 1997
FTD	g.123814T>C	Intronic	Pathogenic	Miyamoto et al., 2001
FTD	g.123815C>T	Intronic	Pathogenic	Takamatsu et al., 1998
FTD	g.123816A>G	Intronic	Pathogenic	Hutton et al., 1998
FTD	g.123817C>T	Intronic	Pathogenic	Lynch et al., 1994
FTD	g.123819C>T	Intronic	Pathogenic	Lanska et al., 1994
FTD	g.123822C>G	Intronic	Pathogenic	Stanford et al., 2003
FTD	g.127672T>G	L650R	Pathogenic	Rosso et al., 2003
FTD	g.127673G>A	L315	Pathogenic	(Bird, 2005, Personal Communication)
FTD	g.127678A>T	K652M	Pathogenic	Zarranz et al., 2005
FTD/PD/MND	g.127687C>T	S655F	Pathogenic	Rosso et al., 2002
FTD	g.132033G>A	G670S	Pathogenic	Spina et al., 2007
FTD	g.132034G>T	G670V	Pathogenic	Neumann et al., 2005
FTD	g.137420G>A	G389R	Pathogenic	Pickering-Brown et al., 2000
FTD	g.137420G>C	G389R	Pathogenic	Murrell et al., 1999
FTD	g.137471C>T	R741W	Pathogenic	Dickson, 1997
FTD	g.137525C>A	Q424K	Pathogenic	(Brice, 2005, Personal Communication)
FTD	g.137535C>T	T762M	Pathogenic	Giaccone et al., 2004
bvFTD	c.163G>A	G55R	Pathogenic	Iyer et al., 2013
FTD	c.363T>C	V363A	Pathogenic	Rossi et al., 2014
FTD	c.363C>A	V363I	Pathogenic	Rossi et al., 2014
FTD	c.454G>A	A152T	Pathogenic	Kara et al., 2012
FTD	c.530A>T	D177V	Unclear	Kim et al., 2014
FTD	c.853A > C	S285R	Pathogenic	Ogaki et al., 2013
FTD	c.892 A>G	K298E	Pathogenic	Iovino et al., 2014
FTD	c.1090C>T	P364S	Pathogenic	Rossi et al., 2012
FTD	c.1096G>A	G366R	Pathogenic	Rossi et al., 2012
FTD	c.1228A>C	N410H	Pathogenic	Kouri et al., 2014
FTD	c.1381-74G > A	Intronic	Pathogenic	Kim et al., 2010a
FTD	c.1908G > A	P636P	Pathogenic	Kim et al., 2010a
FTD	c.1815G > A	P605P	Pathogenic	Kim et al., 2010a
FTD	c.1828-47C > A	Intronic	Pathogenic	Kim et al., 2010a
FTD	c.2002+90G > A	Intronic	Pathogenic	Kim et al., 2010a

(Continued)

TABLE 4 | Continued

Subtypes of dementia	Mutation	Change in amino acid	Type	References
FTD	c.2092G>A	V363I	Pathogenic	Bessi et al., 2010
FTD	IVS10+4A>C	Intronic	Pathogenic	Anfossi et al., 2011
FTD	IVS9-15T>C	Intronic	Pathogenic	Anfossi et al., 2011
FTD	g.132037A>G	Q336R	Pathogenic	Pickering-Brown et al., 2004

FTD, Frontotemporal Dementia; bv FTD, behavioral variant frontotemporal Dementia.
All the information reported in the table is derived from a cumulative study of the literature and the database: http://www.molgen.ua.ac.be/ADMutations/default.cfm?MT=0&ML=2&Page=FTD.

destabilizing a stem-loop structure at the exon-intron junction (D'Souza et al., 1999; Grover et al., 1999; Spillantini and Goedert, 2013). This stem-loop arises as a result of the self complementarity among bases in this region and has a putative role in masking the 5′ splice site Mutations that disrupt the stem-loop structure make the 5′ splice site accesible to splicing factors, leading to inclusion of exon 10 (Wolfe, 2012).

Though mutations lead to alteration of splicing at the mRNA level, their primary effect becomes pathogenic through changes in the protein level in about half of the cases (Goedert and Jakes, 2005).

The human *MAPT* 3′UTR, as well as that of rodents, contains two Polyadenylation Signals (PAS) in tandem and can undergo alternative polyadenylation (APA) to produce transcripts of approximately 2 or 6 kb, namely the short and long transcript variants (Poorkaj et al., 2001). Dickson and colleagues investigated the role of human *MAPT* 3′-UTR in regulating tau expression (Dickson et al., 2013). They observed that the two *MAPT* 3′UTR variants are differentially regulated and influence both mRNA stability and protein expression levels. The same authors have reported that miR-34a can bind the human *MAPT* 3′-UTR long form and reduce tau levels, whereas inhibition of endogenous miR-34 family members leads to increased tau levels, leading to a hypothesis that up-regulation of miR-34 observed during neuronal differentiation could be a compensatory mechanism to decrease the expression of tau aggregates. Recent work (Wu et al., 2013a) also confirms the finding that *MAPT* is regulated by miRNA 34c-5p and miRNA 34c-3p, which bind to its 3′UTR.

Additionally, work by Zovoilis and colleagues have suggested that miR-34c could be a marker for the onset of cognitive disturbances linked to Alzheimers disease and they also indicate that targeting miR-34c could thus be a suitable therapy (Zovoilis et al., 2011).

Studies also reported that miR-34 regulates apoptosis by blocking the SIRT1 gene (Hermeking, 2010) and astrocytic apoptosis has been observed as an early event in FTLD conditions (Broe et al., 2004). These findings suggest that miRNAs might be involved in FTD through apoptotic mechanisms.

Tau is known to spread through synaptic and non-synaptic mechanisms (Medina and Avila, 2014) and its accumulation is thought to be mediated through spreading of the protein from cell to cell. Tau has been reported to be secreted unconventionally in naked form (Chai et al., 2012) or associated to exosomes (Saman et al., 2012) and/or other membrane vesicles (Simón et al., 2012). This method of elimination of tau has been suggested as a response mechanism to inhibit tau secretion and toxicity. Recent reports have suggested that tau is released into culture medium from neuroblastoma cells, tau-expressing non-neuronal cells, induced pluripotent stem cell-derived human neurons, and mouse primary neurons (Kim et al., 2010a; Shi et al., 2012). This has also been observed in the brain interstitial fluid of both wild-type and P301S tau-expressing mice in micro-dialysis studies (Yamada et al., 2011). Clinico-pathological studies underline the tau pathology progression from entorhinal cortex through the hippocampus and into limbic system (Arriagada et al., 1992). Recent *in vivo* studies in tauopathy transgenic mouse models have also highlighted the spreading of tau pathology through a trans-synaptic mechanism in anatomically connected neuronal networks (De Calignon et al., 2012; Liu et al., 2012). Apart from these, intracerebral inoculation of synthetic tau fibrils induced NFT (Neuro fibrillary tangles) like inclusions that propagated from injected sites to other connected regions of brain (Iba et al., 2013).

Current hypotheses also include that pathological progression of improperly folded of tau could be transferred between neuronal cells via a prion-like seeding mechanism which might lead to neurodegeneration.

The major implication observed upon mutations which lead to splice defects highlights the relevance of regulation at RNA level which decides the fate of onset of neurodegeneration. The regulation of MAPT mediated through miRNAs further indicates the role of non-coding RNAs in determining tau protein levels.

GRN (Progranulin)

GRN is located on the long arm of chromosome 17 at the locus q21.31 which is present at a distance of 1.7 Mb from *MAPT* (Baker et al., 2006; Cruts et al., 2006). *GRN* encodes for a 593 aa precursor protein of 68.5 kDa called progranulin.

Structure

Progranulin can be N-glycosylated at five potential sites and secreted as a mature protein of 88 kDa (Chen-Plotkin et al., 2010; Songsrirote et al., 2010). The protein is formed by 7.5 cysteine-rich granulin domains, separated through linker sequences that contain disulfide bridges (He and Bateman, 2003), as represented in **Figure 4B**. This characteristic structure can be cleaved at the intra-linker spacer sequences to produce seven non-identical granulins that contain cysteine-rich motifs. Different proteases can cleave progranulin, such as matrix metalloproteinase-14 (Butler et al., 2008), elastase (Zhu et al., 2002), proteinase 3, and

neutrophil elastase (NE) at the pericellular microenvironment of the neutrophil cell surface (Kessenbrock et al., 2008). The full-length progranulin, once secreted, is protected from cleavage by the high-density lipoprotein (HDL)/Apolipoprotein A-I complex (Okura et al., 2010) and the secretory leukocyte protease inhibitor (SLPI) (Zhu et al., 2002).

Localization and Function

Progranulin is present in many tissues, is highly expressed in immune system cells (Daniel et al., 2000) and in a medium level in the brain (Bhandari et al., 1996; Ahmed et al., 2007), where it is highly expressed in specific populations of neuronal cells, such as cortical neurons, and granule cells of the hippocampus (Daniel et al., 2000; **Table 1**). The subcellular location of progranulin seems to be the endoplasmic reticulum (ER) and Golgi, where it is particular abundant in mouse cortical neurons and mouse microglia (Almeida et al., 2011). Progranulin is implicated in a wide range of biological processes such as embryogenesis (Díaz-Cueto et al., 2000; Daniel et al., 2003; Bateman and Bennett, 2009), cell survival and cell growth (Plowman et al., 1992; He and Bateman, 1999), inflammation and wound repair (Zhu et al., 2002; He et al., 2003; Kessenbrock et al., 2008; Yin et al., 2010), transcriptional repression (Hoque et al., 2003, 2010) and several reports suggest its role in neuronal development (Van Damme et al., 2008). Interestingly, progranulin and the proteolytically cleaved granulins can have coherent functions, such as in the regulation of neurite outgrowth (Van Damme et al., 2008), or they can have contrasting roles, such as in inflammation processes (He and Bateman, 2003).

To date, 69 different *GRN* mutations have been discovered in 231 families (Cruts et al., 2012). A list of detailed pathogenic mutations are reported in **Table 5**. The *GRN* mutations frequency range from 1 to 11.7% in FTD patients, but the frequency rises to 12–25% in familial FTD (Cruts et al., 2006; Gass et al., 2006; Huey et al., 2006; Bronner et al., 2007; Borroni et al., 2008). There are different types of *GRN* mutations, the majority are classified as non-sense, frameshift, and splice site mutations that cause a premature stop codons (Baker et al., 2006; Cruts et al., 2006). However, the pathogenic variants include also missense mutations with a partial decrease of progranulin and a loss of its function (Mukherjee et al., 2006, 2008; Shankaran et al., 2008; Wang et al., 2010). Silent and intronic mutation with unknown pathology can also occur. Generally the pathogenic *GRN* mutations lead to a decreased *GRN* expression due to a non-sense-mediated mRNA decay, resulting in a *GRN* haploinsufficiency inherited in an autosomal dominant manner (Baker et al., 2006; Cruts et al., 2006; Gass et al., 2006; Cruts and Van Broeckhoven, 2008).

Indeed progranulin levels, measured in either the serum or cerebrospinal fluid (CSF) of patients with *GRN* mutations are ∼30–50% of normal (Van Damme et al., 2008). Moreover, a decreased progranulin level can be also detected in plasma of *GRN* mutations patients (Finch et al., 2009) and a reduced GRN mRNA level can be observed in patient whole blood samples through microarray experiments (Coppola et al., 2008). In contrast an increased level of GRN mRNA was observed in the frontal cortex from post-mortem brain samples of FTD patients with *GRN* mutations, as compared to FTD patients without *GRN* mutations (Chen-Plotkin et al., 2010). The higher level of GRN transcripts could be due to the robust microglia infiltrations, observed in the brain tissues of *GRN* mutation patients. Indeed microglia shows high level of GRN expression.

Implications of RNA in Pathogenesis

Most of the patients with FTLD-U show *GRN* mutations with presence of TDP-43 ubiquitin positive inclusions, hence bearing the term FTLD-TDP (Mackenzie et al., 2006, 2010; Sampathu et al., 2006). The relation between TDP-43 and progranulin is not fully understood, however several recent studies indicate that TDP-43 controls the expression of progranulin by binding to GRN mRNA. On a study in which TDP-43 targets were identified through a RIP-chip analysis, it is shown that TDP-43 has a post-transcriptional regulation on GRN and VEGFA (Vascular endothelial growth factor A) (Colombrita et al., 2012).

As previously mentioned, TDP-43 was shown to specifically bind GRN 3′UTR controlling GRN mRNA stability and the final quantity of progranulin protein (Polymenidou et al., 2011; Colombrita et al., 2012). Moreover a knock-down of TDP-43 in mice showed an increase in the amount of GRN mRNA level (Polymenidou et al., 2011; Colombrita et al., 2012). Depletion of TDP-43 also led to altered splicing of sortilin, the putative progranulin receptor (Polymenidou et al., 2011). The relation between GRN and TDP-43 was also demonstrated *in vitro*: cells that were treated with siRNA against GRN for 72 h, showed a caspase-dependent cleavage of TDP-43 into fragments (Zhang et al., 2007); whereas primary neuronal cultures upon knowck-down of GRN showed a re-localization of TDP-43 in the cytoplasm (Guo et al., 2010).

Through genetic association analysis, a common genetic variation localized on the 3′UTR of GRN (rs5848) was shown to represent a genetic risk factor for FTD (Rademakers et al., 2008). Progranulin levels in brain extracts from rs5848 TT homozygous FTD patients were lower than in CC carriers, as observed through western blot analyses, ELISA, and immunohistochemistry. A stronger binding of miR-659 in the 3′UTR of GRN was shown in the presence of the rs5848 T variant, and might explain the reduced progranulin levels.

It is reported that miR-107 is downregulated in presence of Alzheimer's disease at early stage (Wang et al., 2008b). Another study demonstrated through a RIP-Chip analysis performed in human H4 neuroglioma cells that the open reading frame of GRN mRNA contains many recognizing sequences elements for miR-107 (Wang et al., 2010), showing implications of miR-107 deregulation in neurodegenerative diseases. In particular miR-107 regulation of GRN seems to be relevant to glucose metabolism in cultured H4 neuroglioma cells. Previous analysis identified miR-107 as one of the microRNAs that increase their expression with glucose supplementation in cell culture medium (Tang et al., 2009). Wang and colleagues reported that glucose metabolic pathway may recruit miR-107 to regulate GRN expression. Another microRNAs that was found significantly down-regulated in brains of Alzheimer's disease patients

TABLE 5 | List of mutations in *GRN* and their characteristic phenotypes.

Subtypes of Dementia	Mutation	Change in amino acid	Type	References
FTD	delGRN[DR184]	Complete gene deletion	Pathogenic	Gijselinck et al., 2008
FTD	c.-7-20C>T	INTRON	Suggesting Pathogenic	Kim et al., 2010a
FTD	c.349 + 34C > T	INTRON	Suggesting Pathogenic	Kim et al., 2010a
FTD	IVS6+5_8delGTGA	N/A	Unclear	Marcon et al., 2011; Skoglund et al., 2011
FTD	c.1138C>G	Q380E	Unclear	Kim et al., 2014
FTD	g.2988_2989delCA	P439_R440fsX6	Pathogenic	Gabryelewicz et al., 2010
FTD	g.5215A>T	Complete protein degradation	Pathogenic	Le Ber et al., 2007
FTD	g.5217G>C	Complete protein degradation	Pathogenic	Cruts et al., 2006
FTD	g.5913A>G	INTRON	Pathogenic	Mukherjee et al., 2008
FTD/PD	g.8948_12532del	Complete protein deletion	Pathogenic	Rovelet-Lecrux et al., 2008
FTD	g.9044T>C	Predicted failed translation	Pathogenic	Baker et al., 2006
FTD	g.9045G>A	Predicted failed translation	Pathogenic	Cruts et al., 2006
FTD/MND	g.9055G>C	V5L	Unclear	Lopez de Munain et al., 2008
FTD	g.9061T>C	W7R	Unclear	Le Ber et al., 2007
FTD/PPA	g.9068C>A	A9D	Pathogenic	Mukherjee et al., 2006
FTD	g.9132_9133insCTGC	C31LfsX35	Pathogenic	Baker et al., 2006
FTD/PPA	g.9144delC	G35EfsX19	Pathogenic	Gass et al., 2006
FTD	g.9181G>A	Failed translation initiation	Pathogenic	Gass et al., 2006
FTD/AD	g.9319delA	T52HfsX2	Pathogenic	Gass et al., 2006
FTD	g.9399_9400delAG	G79DfsX39	Pathogenic	Gass et al., 2006
FTD	g.9408delC	S82VfsX174	Pathogenic	Bronner et al., 2007
FTD	g.9429G>A	E88	Unclear	Gass et al., 2006
FTD	g.9593T>C	C105R	Unclear	Gass et al., 2006
FTD	g.10129C>T	Q125X	Pathogenic	Baker et al., 2006
FTD	g.10134 C>G	C126W	Unclear	Bernardi et al., 2012
FTD	g.10136_10137delCT	P127RfsX2	Pathogenic	Cruts et al., 2006
FTD	g.10144_10147delCAGT	Q130SfsX125	Pathogenic	Baker et al., 2006
FTD	g.10319G>A	A155WfsX56	Pathogenic	Gass et al., 2006
FTD/PPA	g.10645_10646delCA	S226WfsX28	Pathogenic	Gass et al., 2006
FTD	g.10668C>A	P233Q	Unclear	Bronner et al., 2007
FTD	g.10678C>T	N236	Unclear	Gass et al., 2006
FTD	g.10679G>C	V200GfsX18	Pathogenic	Gass et al., 2006
FTD	g.10965_10966delTG	C253X	Pathogenic	Gass et al., 2006
FTD	g.11002 G>C	A266P	Unclear	Bernardi et al., 2012
FTD	g.11041_11042insCTGA	A237WfsX4	Pathogenic	Cruts et al., 2006
FTD/CBS	g.11240G>C	V279GfsX5	Pathogenic	Gass et al., 2006
FTD	g.11266G>C	E287D	Unclear	Gass et al., 2006
FTD	g.11315_11316insTG	W304LfsX58	Pathogenic	Gass et al., 2006
FTD	g.11316G>A	W304X	Pathogenic	Gass et al., 2006
FTD	g.11339G>A	V279GfsX5	Pathogenic	Baker et al., 2006
FTD/CBS	g.11639delC	T382SfsX30	Pathogenic	Baker et al., 2006
FTD	g.11651G>A	W386X	Pathogenic	Baker et al., 2006
FTD	g.11944_11945delGT	V411SfsX2	Pathogenic	Bronner et al., 2007
FTD	g.11965C>T	R418X	Pathogenic	Baker et al., 2006
FTD	g.12054C>T	H447	Unclear	Bronner et al., 2007
FTD	g.12108_12109insC	C466LfsX46	Pathogenic	Gass et al., 2006
FTD	g.12115C>T	Q468X	Pathogenic	Baker et al., 2006
FTD	g.12227C>T	C474	Unclear	Gass et al., 2006
FTD	g.12282C>T	R493X	Pathogenic	Huey et al., 2006
FTD	g.12428G>C	W541C	Unclear	Bronner et al., 2007

AD, Alzheimers disease; CBS, Corticobasal syndrome; FTD, Frontotemporal Dementia; PPA, Primary progressive aphasia; MND, Motor neuron disease; PD, Parkinsons disease. All the information reported in the table is derived from a cumulative study of the literature and the database: http://www.molgen.ua.ac.be/ADMutations/default.cfm?MT=0&ML=2&Page=FTD.

is miR-29b, that beloged to the miR-29a/b-1 cluster (Hébert et al., 2008). Interestingly progranulin can also be regulated by miR-29b through a binding in the 3′UTR of GRN mRNA (Jiao et al., 2010). It would be useful to know if these microRNAs deregulation can contribute to the pathogenesis of dementia. So far different microRNAs seem to be important for the control of progranulin along with the role played by TDP-43 on the stability of GRN mRNA and its expression.

VCP

The VCP (Valosin-containging protein) gene is located on chromosome 9p13.3. It also called p97 or CDC48, consists of 17 coding exons.

Structure

The VCP protein is composed of four domains vital for its proper functioning, namely the N, D1 D2 and C-terminal domains (**Figure 5B**; DeLaBarre et al., 2006; Pye et al., 2007). The VCP N domain is encoded by exons 1, 2, 3, 4 and 5, while the D1 and D2 domains are encoded by exons 6, 7, 8, 9, 10 and 12, 13, 14, respectively. There are two linker domains in the protein: the N-D1 linker and the flexible D1-D2 linker.

VCP is a member of the AAA-ATPase gene superfamily (ATPase Associated with diverse cellular Activities) (Woodman, 2003; Wang et al., 2004b), and is one of the most abundant cytosolic proteins (**Table 1**) conserved throughout in mammals. The complete protein contains 806 amino acids. The N domain of VCP is responsible for the cofactor and ubiquitin binding

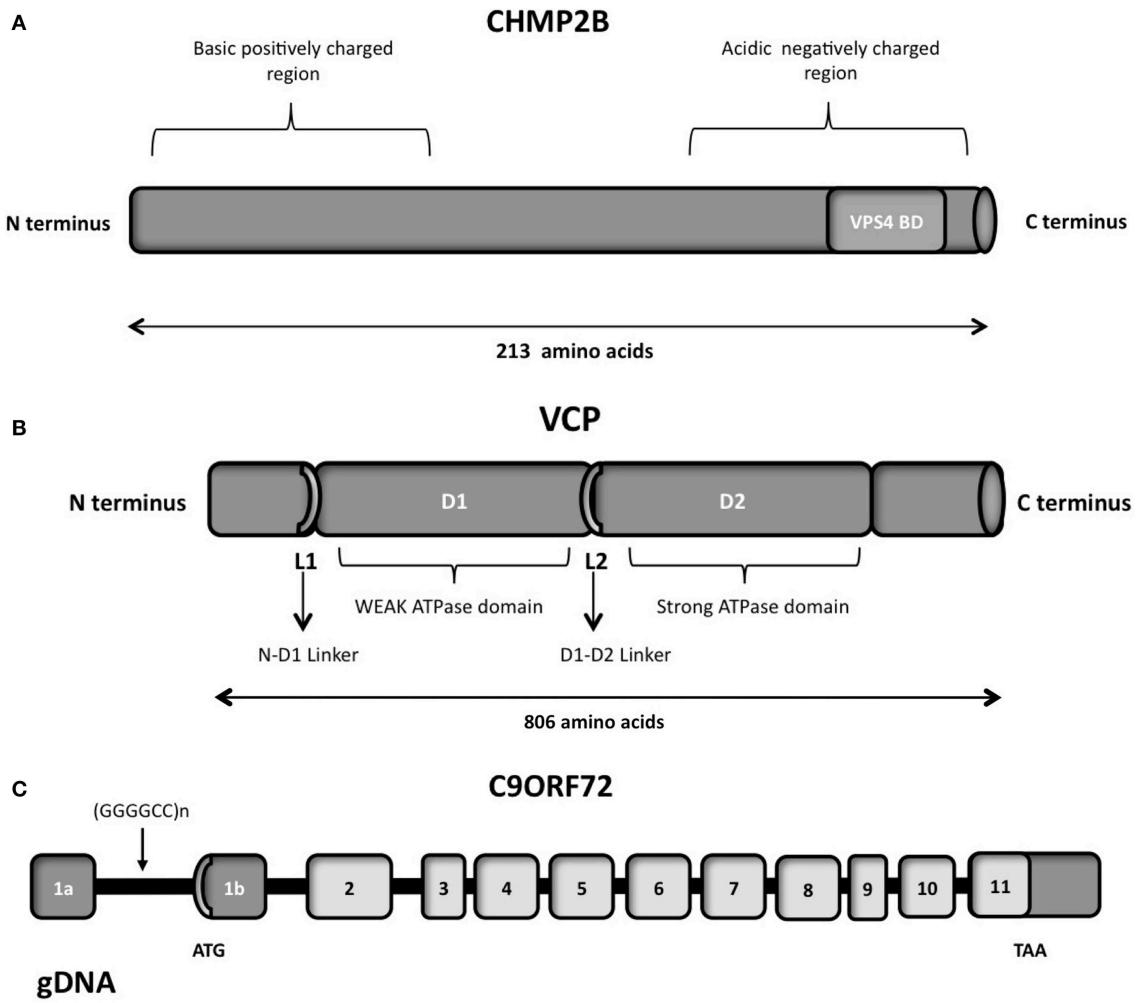

FIGURE 5 | (A) Schematic representation of the CHMP2B which contains an acidic negatively charged C-terminal domain and a basic positively charged N-terminal domain, a predicted coiled-coil structure (14-51 aa) and a conserved Snf7 domain (16-178 aa). The autoinhibitory structure formed in the cytosol due to the C- and N- terminal part interactions is reverted through the binding of VSP4 on the VPS4 binding domain (VPS4 BD), localized on the C-terminal part. **(B)** Schematic representation of the six functional domains of the VCP protein: the N-terminal domain, the weak ATPase domain (D1), the major ATPase domain (D2), the N-D1 linker domain, the flexible D1–D2 linker domain and the C-terminal domain. **(C)** Overview of the genomic structure of the *C9ORF72* gene, with white boxes representing the coding exons and gray boxes representing the non-coding exons. The position of the hexanucleotide repeat (GGGGCC), the start codon (ATG), and the stop codon (TAA) are indicated in the scheme.

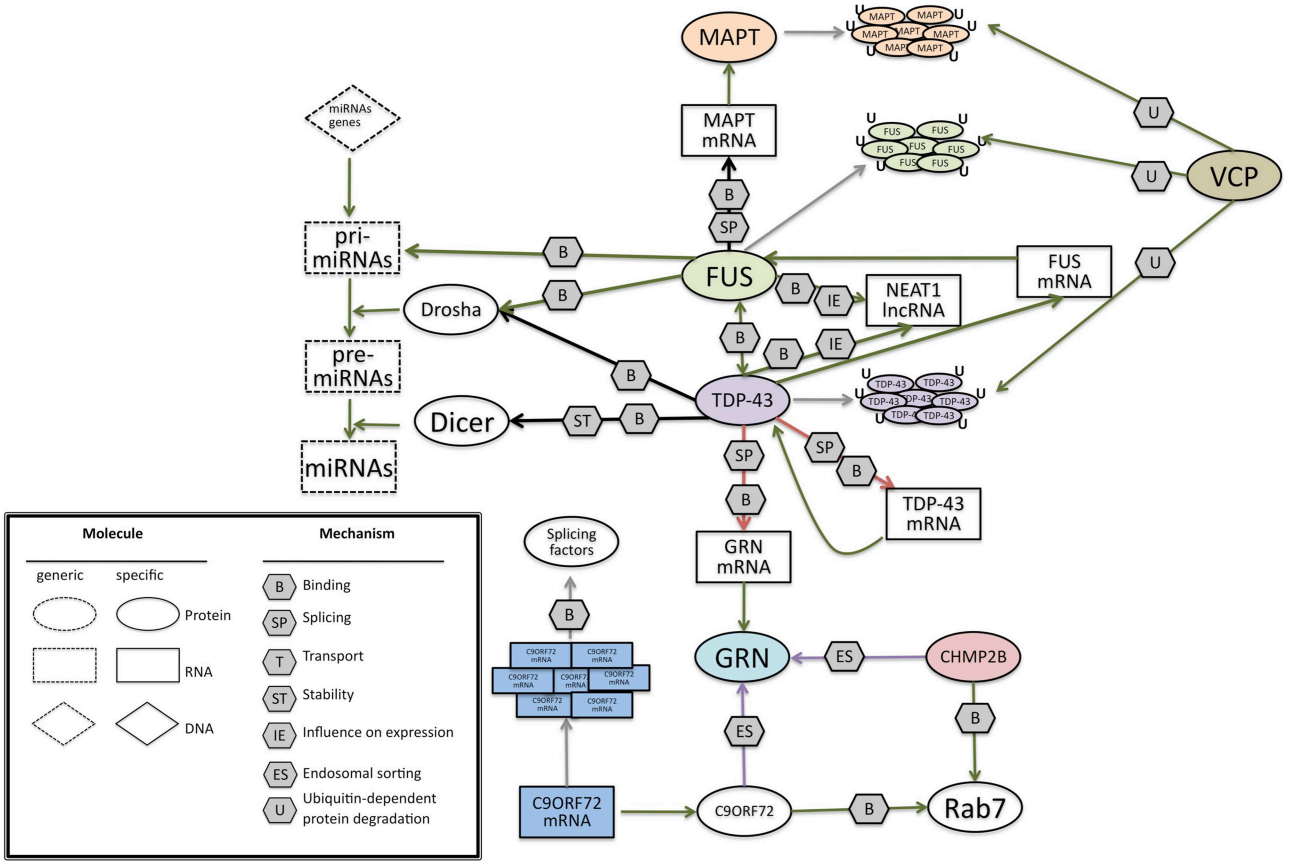

FIGURE 6 | A possible network of interactions between proteins and RNAs, at the basis of FrontoTemporal Dementia. Green arrows indicate binding interactions or processes that result in activation or increased expression. Red arrows indicate binding interactions or processes that result in inhibition of activity or reduced expression. Black arrows indicate binding interactions or processes whose result can be either positive or negative. Purple arrows indicate binding interactions or processes which are hypotetical. Symbols as in Legend. lncRNAs, long non-coding RNA; TDP-43, TAR DNA binding protein; FUS, Fused in Sarcoma; GRN, progranulin; MAPT, Microtubule-Associated Protein Tau; VCP, Valosin Containing Protein; C9ORF72. CHMP2B, Charged multivesicular body protein 2b; Rab7, Ras-related protein 7.

function (Wang et al., 2004b). While the D1 domain mediates oligomerization-independent nucleotide binding, the D2 domain confers most of the ATPase activity (Wang et al., 2004b).

Localization and Function

This protein functions as a molecular chaperone in various distinct cellular processes including ubiquitin-dependent protein degradation, stress responses, programmed cell death, nuclear envelope reconstruction, and Golgi and endoplasmic reticulum (ER) assembly (Guinto et al., 2007).

VCP is known to be involved in protein aggregation/quality control of mitochondria and cell proliferation (Hayashi, 2013) and is vital for retro-translocation of misfolded proteins from Endoplasmic reticulum to cytoplasm (Kimonis et al., 2008). Mutation and depletion studies of VCP have provided evidence of accumulation of poly-ubiquitinated proteins (Dai and Li, 2001). Mutations in this gene may suggest the disruption of normal protein degradation pathway in the disease. This could be facilitated through the disruption of binding between the VCP and protein adaptors.

The expression of mutant VCP in myoblastic cell lines is associated with increased ubiquitin conjugated proteins (Weihl et al., 2006). Studies on overexpression of mutant VCP protein in transgenic mice implicated an age-dependent muscle weakness and Ubiquitin-positive inclusions and accumulation of high molecular weight protein aggregates (Weihl et al., 2007).

VCP functions as a homohexamer (Zhang et al., 2000; Rouiller et al., 2002) by binding to multiple proteins associated with Ubiquitin proteasome system (UPS). The VCP complex binds to polyubiquitin chains and unbounds ubiquitinated proteins from their binding partners thereby facilitating transport to the UPS.

Implications of RNA in Pathogenesis

To date, 18 different VCP mutations have been discovered in 48 different families, which include FTLD that is associated with ALS, inclusion body myopathy, and Paget disease (Cruts et al., 2012). **Table 6** high-lights the list of pathogenic mutations observed so far. The association of inclusion body myopathy and FTD was established by Kovach et al. (2001).

TABLE 6 | List of mutations in *VCP* and their characteristic phenotypes.

Subtypes of Dementia	Mutation	Change in amino acid	Type	References
IBMPFD	g.284G>A	R92H	Unclear	Kaleem et al., 2007
IBMPFD	g.410C>T	P137L	Pathogenic	Stojkovic et al., 2009
IBMPFD	g.4438C>T	R93C	Pathogenic	Guyant-Maréchal et al., 2006
IBMPFD	g.4444C>G	R95G	Pathogenic	Watts et al., 2004
IBMPFD	g.4444C>T	R95C	Pathogenic	Kimonis et al., 2008
IBMPFD	g.6990C>T	R155C	Pathogenic	Watts et al., 2004
IBMPFD	g.6991G>A	R155H	Pathogenic	Watts et al., 2004
IBMPFD	g.6991G>T	R155L	Unclear	Kumar et al., 2010
IBMPFD	g.6991G>C	R155P	Pathogenic	Watts et al., 2004
IBMPFD	g.6990C>A	R155S	Pathogenic	Stojkovic et al., 2009; Vesa et al., 2009
IBMPFD	g.6996G>C	G157R	Pathogenic	Stojkovic et al., 2009; Djamshidian et al., 2009
IBMPFD	g.7002C>T	R159C	Pathogenic	Bersano et al., 2009
IBMPFD	g.7003G>A	R159H	Pathogenic	Haubenberger et al., 2005
IBMPFD	g.7099G>A	R191Q	Pathogenic	Watts et al., 2004
IBMPFD	g.8085T>G	L198W	Pathogenic	Watts et al., 2007
IBMPFD	g.8187C>A	A232E	Pathogenic	Watts et al., 2004
IBMPFD	g.9349A>G	T262A	Pathogenic	Spina et al., 2008
IBMPFD	g.10742A>C	N387H	Pathogenic	Watts et al., 2007
IBMPFD	g.11295G>C	A439P	Pathogenic	Shi et al., 2012
IBMPFD	g.11295G>T	A439S	Pathogenic	Stojkovic et al., 2009

IBMPFD, Inclusion body myopathy with Paget's disease of the bone and frontotemporal dementia.
All the information reported in the table is derived from a cumulative study of the literature and the database: http://www.molgen.ua.ac.be/ADMutations/default.cfm?MT=0&ML=2&Page=FTD.

A recent work by Jacquin et al. (2013) has showed R155H (464 G>A) mutation of the *VCP* gene in a French family, led to the Inclusion body myopathy with Paget's disease of the bone and frontotemporal dementia (IBMPFD), with a psychiatric onset of FTD.

The expression of IMBPFD-associated *VCP* mutations in skeletal muscle cells reduced UNC-45 (a molecular chaperone involved in myosin assembly) degradation that is linked to severe myofibril disorganization in myotubules. This study suggests a possible mechanism for the selective vulnerability of skeletal muscle in IBMPFD; however, the implication for the pathogenesis of FTD still remains unknown. Studies on a *VCP*-mutant transgenic mouse have shown TDP-43 and ubiquitin positive accumulations (Custer et al., 2010) suggesting a possible interplay between these proteins. IBMPFD is known to have TDP-43 aggregation with *VCP* mutations (Nalbandian et al., 2011). Ju et al. (2009) have established a link between *VCP* and autophagosomes wherein the loss of VCP leads to accumulation of autophagosomes, thus establishing a possible cause of aggregation of proteins such as TDP-43.

VCP has been detected in a few inclusions of neurodegenerative diseases such as senile plaques in Alzheimer's disease, Lewy bodies in Parkinson's disease, neuronal intranuclear inclusions in CAG/polyglutamine diseases and ubiquitin-positive inclusions in ALS (Hirabayashi et al., 2001; Mizuno et al., 2003; Ishigaki et al., 2004).

Bartolome and colleagues have performed analyses in fibroblasts derived from patients with three different pathogenic *VCP* mutations, VCP-deficient cells, mouse cortical primary neurons and astrocytes, to conclude that reduction of VCP led to uncoupling of mitochondria and increased oxygen consumption and a subsequent decrease in ATP of cells leading to cellular toxicity and neuronal death (Bartolome et al., 2013).

VCP has been recently involved in clearance of mRNP granules (Buchan et al., 2013), thereby unraveling a new mechanism in clearance of RNPs from the cell. This might indicate why *VCP* mutations lead to accumulation of stress granule constituents or cytoplasmic inclusions. mRNP granules assemble to form stress granules as a consequence of their aggregation (Erickson and Lykke-Andersen, 2011). Wang et al. (2015) have shown a direct interaction between VCP and FUS. VCP being a key regulator of protein degradation, DNA interaction, and mitochondrial activity, its direct interaction with FUS is intriguing. Although there is no evidence which shows a direct interaction or implication of *VCP* mutations on RNA, its association with TDP-43 and FUS, both of which are RNA binding proteins may suggest their unraveled interactions in RNA metabolism.

CHMP2B

CHMP2B (Charged multivesicular body protein) is encoded by a gene located on chromosome 3 and is a component of the endosomal sorting complex required for transport-III (ESCRT-III complex).

Structure

CHMP2B is a protein of 213 amino acids, with an acid C-terminal domain and basic N-terminal domain (**Figure 5A**), and

a predicted coiled-coil structure (Skibinski et al., 2005). The negatively charged C-terminal domain interacts with the positively charged N-terminal part, creating a closed and autoinhibitory structure in the cytosol (Whitley et al., 2003; Shim et al., 2007). CHMP2B requires therefore an activation process performed by VPS4, which binds to its C-terminal domain. Indeed the C-terminal part of CHMP2B contains a binding domain for VPS4. VPS4 is a member of the AAA-ATPase family and it has a role in catalyzing the dissociation of ESCRT complexes from endosomes (Katzmann et al., 2002). The ATPase activity of VPS4 is important for endosomal sorting (Katzmann et al., 2002; Obita et al., 2007). Mutations localized in the VSP4-binding region impair the function of CHMP2B, preventing the formation of protrusions from the cell (Bodon et al., 2011).

Localization and Function

Northern-Blot analysis demonstrated that CHMP2B is expressed in all the major parts of the brain, including the temporal and frontal lobes (**Table 1**). Moreover through *in situ* hybridization of mouse brain, an enhanced expression of CHMP2B in the hippocampus, frontal and temporal lobes, and cerebellum was shown (Skibinski et al., 2005). The endosomal-sorting complex required for transport (ESCRT) is a protein complex involved in the endocytosed protein trafficking from endosome to lysosomes, playing an important role for sorting of proteins and for their efficient lysosomal degradation (Urwin et al., 2010). Moreover ESCRT complexes have a relevant role at the plasma membrane, during cytokinesis (Carlton and Martin-Serrano, 2007; Elia and Sougrat, 2011), budding of some enveloped viruses (Usami et al., 2009; Martin-Serrano and Neil, 2011), autophagy and transcriptional regulation (Roxrud et al., 2010; Schmidt and Teis, 2012). Endosomal trafficking is a key process for the internalization and transport of neuronal growth factors, secreted growth factors, signaling molecules (Bronfman et al., 2007). A dysfunction in this process could lead to an altered cell-signaling and aberrant communication between cells.

Implications of RNA in Pathogenesis

In particular ESCRT dysfunction is associated with neurodegeneration, indeed mutation in *CHMP2B* have been reported in frontotemporal dementia linked to chromosome 3 (FTD-3) (Urwin et al., 2010). FTD-3 has an onset of 48–67 years and is an autosomal dominant dementia with TDP-43 negative FTLD-U, ubiquitin positive inclusions (Urwin et al., 2010).

As reported in **Table 7**, several missense mutations are connected with FTD-3 (Skibinski et al., 2005), with familial or sporadic cases of ALS (Parkinson et al., 2006; Urwin et al., 2010), familial frontotemporal lobar degeneration (FTLD) (Ghanim et al., 2010) or CBD (Van der Zee et al., 2008), however only few studies analyzed their pathogenic features in cultured neurons.

A point mutation has been identified in the 5′ acceptor site of exon 6, causing the production of two abnormal transcripts: $CHMP2B^{intron5}$ with retention of intron 5 and $CHMP2B^{\Delta 10}$ that has a 10 bp deletion and a sequence frameshift due to the use of a criptic splice site in exon 6 (Skibinski et al., 2005). Both proteins lack 36 amino acids in the C-terminal part with the subsequent absence of VPS4-binding domain and an accumulation of CHMP2B on the endosomal membrane (Skibinski et al., 2005; Urwin et al., 2010). This accumulation suggests that the binding of mutated proteins to the endosomes prevents the recruitment of the correct proteins necessary for the fusion with lysosomes (Metcalf and Isaacs, 2010; Urwin et al., 2010). Indeed large and abnormal endosomal structures are observed in post-mortem brain tissues, in patient fibroblast cultures and in case of overexpression of CHMP2B mutant in PC12 and human neuroblastoma cell lines (Skibinski et al., 2005; Van der Zee et al., 2008; Urwin et al., 2010). Moreover CHMP2B seems to act through a gain of function mechanism in the presence of mutations, since only the transgenic mice expressing $CHMP2B^{intron5}$ show similar neuropathology features observed in FTD-3 patients, whereas the knockout mice with the inactivation of CHMP2B do not show any pathological characteristics (Ghazi-Noori et al., 2012).

Another non-sense mutation replaces a glutamine residue with a stop codon, creating a more severe C-terminal truncation (Van der Zee et al., 2008).

TABLE 7 | List of mutations in CHMP2B and their characteristic phenotypes.

Subtypes of Dementia	Mutation	Change in amino acid	Type	References
FTD	g.13227A>G	I29V	Unclear	Cannon et al., 2006
FTD	g.18376C>A	T104N	Unclear	Cox et al., 2010
FTD	g.25885A>G	N143S	Unclear	Van der Zee et al., 2007
FTD	g.25899G>T	D148Y	Pathogenic	Skibinski et al., 2005
FTD	g.25950C>T	Q165X	Pathogenic	Van der Zee et al., 2007
FTD	g.26189G>C	p.M178VfsX2/p.M178LfsX30	Pathogenic	Skibinski et al., 2005
FTD	g.26214C>T	R186X	Unclear	Momeni et al., 2006
FTD	g.26218G>A	S187N	Unclear	Ferrari et al., 2010
FTD	g.26276A>C	Q206H	Pathogenic	Parkinson et al., 2006
FTD	c.581C>T	S194L	Unclear	Ghanim et al., 2010

FTD, Frontotemporal Dementia.

All the information reported in the table is derived from a cumulative study of the literature and the database: http://www.molgen.ua.ac.be/ADMutations/default.cfm?MT=0&ML=2&Page=FTD.

Since CHMP2B is involved in the endosomal trafficking of signal molecules, it could be interesting and possibly relevant for the pathology to check if an altered endosomal process can affect the function of other proteins involved in FTLD, such as progranulin, as is it also suggested by Urwin et al. (2010).

C9ORF72

Structure

Large expansions of the non-coding GGGGCC repeat in *C9ORF72* (Chromosome 9 open reading frame 72) were recently demonstrated to cause ALS and FTD (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Indeed 20–80% of familial and 5–15% of sporadic ALS and FTD in North American and European patients show this hexanucleotide expansion with a range of 700–1600 repeats, whereas the normal population carries less than 30 repeats (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Smith et al., 2012). Pathogenic mutations reported in *C9ORF72* are listed in **Table 8**. *C9ORF72* is localized on chromosome 9 and is composed of 12 exons, with two alternate non-coding first exons (**Figure 5C**, exon 1a and 1b) (DeJesus-Hernandez et al., 2011). Specifically, the polymorphic hexanucleotides repeat was identified between the two alternatively spliced non-coding exons, through a sequencing approach (DeJesus-Hernandez et al., 2011).

Depending on alternative transcription initiation, the GGGGCC repeat can be located on the promoter of transcriptional variant 1 or in the intron 1 of transcriptional variants 2 and 3. Variant 2 results from splicing of exons 1a and exons 2–5 whereas variant 3 is composed of exon 1a and exons 2–11.

Localization and Function

Expression array data showed wild type C9ORF72 RNA present in different CNS tissues, including spinal cord and higher expression in the cerebellum (Renton et al., 2011; **Table 1**).

The protein encoded by *C9ORF72* is still uncharacterized and with unknown function even if it is well-conserved across species (DeJesus-Hernandez et al., 2011).

Recently, Farg et al. (2014) demonstrated for the first time that the endogenous C9ORF72 protein has a function in the regulation of intracellular trafficking processes in the endosomal and autophagy-lysosomal compartments in neuronal cell lines. Therefore, they reported the normal cellular function of C9ORF72 that is essential to understand its role in FTD/ALS.

In particular, they found co-localization in neuronal cell lines and primary cortical neurons of C9ORF72 with four Rab proteins, which are involved in endosomal trafficking. In motor neurons, they found 70% of colocalization with Rab7, which is a fundamental protein implicated in the biogenesis of lysosomes, the transport of endosomes and the maturation of autophagosomes (Gutierrez et al., 2004). A similar mechanism of interaction and recruitment of Rab7 was also described for CHMP2B by Urwin and collaborators. In *CHMP2B* mutant cells, an impaired recruitment of Rab7 onto endosomes was observed with a decreased fusion with lysosomes and a delayed degradation (Urwin et al., 2010).

C9ORF72 protein was also found to colocalize with lysosomes, ubiquilin-2 and autophagosomes, involved in autophagy (Farg et al., 2014). Interestingly, the ability of C9ORF72 to interact with hnRNPs and induce not yet characterized nuclear aggregates and stress granules, could link the C9ORF72 protein with RNA metabolism processes (Farg et al., 2014).

Implications of RNA in Pathogenesis

Immunocytochemistry analysis on human fibloblasts and mouse motor neuron NSC-34 cell line revealed a predominant nuclear localization of C9ORF72 protein (Renton et al., 2011). Immunohistochemical analysis showed C9ORF72 expression in neurons and in FTD- and ALS-affected regions with a predominant cytoplasmic staining and a synaptic localization, but the quantitative mRNA analysis demonstrated that the repeat expansion reduces C9ORF72 transcript variant 1 expression in lymphoblast cell lines of expanded repeats carriers and in frontal cortex samples from unrelated FTLD-TDP patients carrying expanded repeats (DeJesus-Hernandez et al., 2011). The hexanucleotide repetitions present in the C9ORF72 transcript can form G-tetrad units, called G-quartets, where G bases are rearranged in a cyclic pattern with eight hydrogen bonds (Fratta et al., 2012). The presence of RNA G-quadruplexes has been found in different organisms and has been observed *in vitro* and *in vivo* (Kikin et al., 2008; Xu et al., 2010b). Transcripts are enriched in RNA G-quadruplexes structures in the 5′UTR, 3′UTR and in the first exon (Eddy and Maizels, 2008; Huppert et al., 2008). Recently a molecular mechanism was described by which the DNA and RNA G-quadruplexes in C9ORF72 create structures that promote the formation of RNA/DNA hybrids (R-loops) (Haeusler et al., 2014). The pathological mechanism involving*C9ORF*72 gene or its function are not clear, even if several studies showed a decrease in the mRNA levels of some C9ORF72 variants in ALS, which suggests a loss-of-function mechanism (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Gijselinck et al., 2012; Mori et al., 2013b). Moreover, the aberrant transcripts containing

TABLE 8 | List of mutations in *C9ORF72* and their characteristic phenotypes.

Subtypes of Dementia	Mutation	Change in amino acid	Type	References
FTD/ALS	g.5321GGGGCC(?)	G4C2 hexanucleotide repeat expansion	Pathogenic	DeJesus-Hernandez et al., 2011
FTD	g.11942A>T	T66S	Pathogenic	Van der Zee et al., 2013

TD, Frontotemporal Dementia; ALS, Amyotrophic lateral sclerosis.

All the information reported in the table is derived from a cumulative study of the literature and the database: http://www.molgen.ua.ac.be/ADMutations/default.cfm?MT=0&ML=2&Page=FTD.

the hexanucleotide repeats can accumulate and form structures in the nucleus called RNA foci, which may produce neurodegeneration through a toxic effect (DeJesus-Hernandez et al., 2011). These transcripts can be aberrantly expressed through repeat-associated non-ATG (RAN) translation (Mori et al., 2013b). Several groups reported that the RAN translation of the hexanucleotide repeats produces poly(GP), poly(GA) and poly(GR) proteins, since this type of translation, without an initiation codon, can have all the possible reading frames (Ash et al., 2013; Mori et al., 2013b) and RNA can be also bidirectionally transcribed (Gendron et al., 2013). These RAN proteins can form inclusions in neurons and are considered a hallmark of the disease (Ash et al., 2013; Mori et al., 2013b). The neuronal inclusions can be detected through antibodies that recognize putative GGGGCC repeat RAN-translated peptides, therefore this type of immunoreactivity can be use as a potential biomarker for the disease (Ash et al., 2013).

It is also reported that RNA foci may sequester important RNA binding proteins, causing an alteration inside the cell and a subsequent dysfunction of RNA targets, in a process similar to the formation of RNA foci in myotonic dystrophy type 1 (DM1) (Lee et al., 2013; Mori et al., 2013a; Reddy et al., 2013; Xu et al., 2013). Specifically, one study demonstrated that hnRNP-H is sequestrated by RNA foci, reducing its available amount and its splicing efficiency on different target transcripts (Lee et al., 2013). A recent paper by Gendron et al. (2013) contains detailed descriptions of the proteins found to be sequestered on RNA foci in *in vitro* studies.

The presence of both sense and antisense RNA foci in frontotemporal dementia with the presence of *C9ORF72* repeats (C9FTLD), was demonstrated in patients, specifically in the frontal cortex, motor cortex, hippocampus, cerebellum, and spinal cord (Gendron et al., 2013; Lagier-Tourenne et al., 2013; Mizielinska et al., 2013; Zu et al., 2013). RNA foci were identified in neurons and with lower frequency in astrocytes, microglia, and oligodendrocytes; the highest concentration of foci was found in the frontal cortex region, compared to cerebellum and hippocampus (Mizielinska et al., 2013). However, another work reported that foci are localized with higher frequency in the cerebellum (Lee et al., 2013). Despite this inconsistency, the major part of RNA foci is localized at the very edge of the nucleus, but the explanation for this localization is still unknown (Mizielinska et al., 2013). The cellular toxicity associated with the longer hexanucleotide repeats and the presence of RNA foci was demonstrated using neuroblastoma cells and zebrafish embryos (Lee et al., 2013). One patient, who was homozygous for the *C9ORF72* hexanucleotide repeats, showed a higher proportion of sense and antisense foci with an early onset of FTD and severe pathological characteristics, compared to the heterozygous case (Mizielinska et al., 2013). A recent study found a mechanism for the disease in which the DNA and RNA–DNA structures formed in the repeat regions, alter the RNA transcription, with a result of transcriptional pausing and abortion. The accumulation of abortive transcripts with hexanucleotides repeats, creates G-quadruplexes, and hairpins structures with a binding of essential proteins, leading to nuclear stress, and further defects (Haeusler et al., 2014).

TDP-43 and FUS, two FTD related proteins previously decribed, are structurally related to the hnRNPs that are found to bind C9ORF72 RNA foci (Lee et al., 2013; Mori et al., 2013b), however FUS and TDP-43 do not colocalize with C9ORF72 RNA foci in cells, patient motor neuron cultures or in spinal motor neurons from patients (Lagier-Tourenne et al., 2013; Lee et al., 2013; Sareen et al., 2013). Since TDP-43 is capable to bind through its C-terminal region the hnRNP proteins (Buratti et al., 2005), the accumulation of these ribonucleoprotein on the RNA foci could indirectly influence TDP-43 function, creating a possible link of interaction between these two factors involved in FTD and ALS (Gendron et al., 2013). Indeed most of the cases with *C9ORF72* expansion show TDP-43 inclusions (FTLD-TDP) (DeJesus-Hernandez et al., 2011; Lagier-Tourenne et al., 2013; Mackenzie et al., 2014) with some exception, such as a case in UK with *C9ORF72* repeats with FTLD-tau pathology (Snowden et al., 2012). It was reported that the plasma and CSF level of phosphorylated TDP-43 is significantly higher in patients with FTD carrying *C9ORF72* expansion or *GRN* mutations compared to other FTD patients or healthy controls (Suárez-Calvet et al., 2014). This finding creates another possible link of interaction or regulation between TDP-43 and C9ORF72 that needs further analysis.

Discussion

In this review we describe the different genes involved in FTD, focusing on their possible interactions, in order to identify a common network of their combined regulations. We created this network focusing on the RNA aspect, an emerging and crucial molecule that plays critical and fundamental functions in the cells. Recently, research has increased its focus onthe role of RNA in neurodegeneration (Renoux and Todd, 2012). We believe that the RNA mediated regulation plays a key role in the unique integration of all the known genes involved in FTD.

In this picture (**Figure 6**) FUS and TDP-43 RNA binding proteins are at the core of the network, since they often are associated factors that share similar features, with sometimes different but complementary roles (Colombrita et al., 2012). They interact with RNA in three main roles: as RNA binding proteins participating on the different aspects of mRNA processing (Bosco et al., 2010; Colombrita et al., 2011), as regulators of microRNAs processing, and as regulators of lncRNAs. FUS and TDP-43 were both found in aggregates in ALS/FTLD affected neurons (Da Cruz and Cleveland, 2011), nuclear complexes and in cytoplasmic RNPs (Kim et al., 2010b). TDP-43 appears to be the main regulator of this network, being able to interact with FUS pre-mRNA and regulate its splicing, and auto-regulate its own pre-mRNA, causing a reduction of its own expression (Polymenidou et al., 2011).

TDP-43 can also bind GRN pre-mRNA, negatively controlling its splicing and, accordingly, knock-down of TDP-43 was shown to increase the amount of GRN mRNA level (Polymenidou et al., 2011; Colombrita et al., 2012). In the presence of *GRN* mutations, TDP-43 regulation can be altered, causing the formation of inclusions containing TDP-43 (Mackenzie et al., 2006, 2010; Sampathu et al., 2006). Though TDP-43 aggregation is a typical hallmark of many other neurodegenerative disorders, such as Alzheimer's

disease, Guam Parkinsonism dementia complex, and Lewy body disease (Dickson, 2008), its impact on FTD in influencing the regulation of the network should not be underestimated.

On the other side, FUS acts as a splicing regulator of MAPT mRNA, indeed it was demonstrated that silencing of FUS alters the splicing of MAPT. In particular, FUS helps the skipping of exon 10 in primary cortical neurons (Ishigaki et al., 2012). The presence and the absence of exon 10 in *MAPT* gene has a fundamental role in the regulation of a delicate balance in the ratio of 4 or 3 repeats that can lead to FTD.

For the recently identified *C9ORF72* gene, large expansions of the non-coding GGGGCC repeat correlate with pathogenesis, making an RNA gain-of function mechanism possible. Indeed the aberrant C9ORF72 transcripts accumulate in nuclear RNA foci and sequester several RNA-binding proteins, including some splicing factors. However, other possible pathogenetic mechanisms are under scrutiny for *C9ORF72*, including impaired transcription of the expanded gene or repeat-associated non-ATG (RAN) translation of expanded transcripts.

In this scenario, two FTD genes code for proteins that fit in the picture not for their relation to RNA, but for their role in protein degradation.

VCP, taking part in the ubiquitin-proteasome pathway and protein turnover (Zhang et al., 2000; Rouiller et al., 2002), could be involved in the degradation of protein inclusions present in different forms of FTD. TDP-43 inclusions were found in the presence of a *VCP* mutation (Neumann et al., 2009a). A direct interaction between VCP and FUS has been observed suggesting a possible convergence in their functions (Wang et al., 2015).

CHMP2B regulates protein trafficking between endosomes and lysosomes and is involved in the protein degradation pathway through lysosomes (Urwin et al., 2010). Therefore, CHMP2B could be relevant for the internalization and transport of neuronal growth factors or signaling molecules such as progranulin.

Recently, a function for the C9ORF72 protein was uncovered, in the regulation of intracellular trafficking processes in the endosomal and autophagy-lysosomal compartments (Farg et al., 2014), providing an additional link to VCP and CHMP2B proteins.

During the preparation of this review a recent study performed by Van der Zee and colleagues have demonstrated ananalysis on a European cohort of 1808 FTLD patients revealing mutationsin SQSTM1 (Sequestosome 1) or p62. The p62 protein is a stress-responsiveubiquitin-binding protein, which is shown to have a role in degradation ofpolyubiquitinated proteins via the proteasome pathway or autophagicprocesses (Van der Zee et al., 2014). This gene was known to be associated with ALS and found as a rare mutation with a frequency of 1–3% in both ALS and FTLD cases. This further intrigued its possible role in pathogenicity with a common patho-mechanism. p62 is present in neuronal and glial ubiquitin-positive inclusions in different tauopathies and synucleinopathies (Van der Zee et al., 2014). The meta-analysis performed by Van der Zee and colleagues revealed that rare mutations clustering in the UBA domain of SQSTM1 may influence disease susceptibility by doubling the risk for FTLD. Further, histopathology analysis of autopsied brain of SQSTM1 mutation carriers demonstrated a widespread of neuronal and glial phospho-TDP-43 pathology. Therefore, this study opens up another target gene SQSTM1, which is known to have implications in FTLD/ALS and additionally associated with TDP-43. Despite further work being needed, to unravel and confirm the details of the proposed network, we foresee that the construction of a picture of the interactions between proteins and RNAs at the basis of the FTD pathology will be of invaluable importance, not only to comprehend the pathogenetic mechanisms but also to develop new and more effective therapeutical approaches. Through the network analysis proposed in this review, it can be foreseen that more genes can be linked to FTD and their roles will possibly fall in to two main categories: regulation of gene expression through RNA or protein degradation. Additionally it could be predicted that novel genes related to FTD in future will be possibly a part of the proposed network.

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Circulating cell-free microRNAs as biomarkers for neurodegenerative diseases

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1. Introduction

Neurodegenerative diseases include a range of debilitating conditions of the central nervous system characterized by the progressive loss of neural tissues. More than 600 disorders afflict the nervous system, as hereditary or sporadic conditions that progressively cause neurodegeneration.
The main neurodegenerative diseases are:
Alzheimer's disease (AD) and other dementias (FTD);
Parkinson's disease (PD) and PD-related disorders;
Huntington's Disease (HD);
Amyotrophic Lateral Sclerosis (ALS).
These diseases are multifactorial debilitating disorders of the nervous system accounting for a significant and increasing proportion of mortality in the world. All these neurodegenerative diseases are characterized by loss and death of neurons in specific brain areas and by different clinical manifestations.
Alzheimer disease (AD) is the most common cause of dementia. It is characterized by cortical atrophy and loss of neurons in the parietal and temporal lobes. It is a progressive neurodegenerative disorder characterized by memory impairment with executive dysfunction, motor problems, and/or language difficulties. AD is caused by mutations in three highly penetrant genes: Amyloid Precursor Protein (*APP*), Presenilin 1 (*PSEN1*) and

Presenilin 2 (*PSEN2*) (Levy-Lahad et al., 1995) and one susceptibility gene (*APOE*) (Strittmatter et al., 1993).

Frontotemporal dementia (FTD) is characterized by atrophy of the brain that predominantly affects the frontal and temporal lobes, and is often used as a synonym for Fronto-Temporal Lobar Degeneration (FTLD). It results in progressive behavioral changes and language dysfunction, but motor and cognitive impairment may be present as well. Mutations in five genes have been linked to FTLD: Microtubule-Associated Protein Tau (*MAPT*) (Hutton et al., 1998), Progranulin (*GRN*) (Baker et al., 2006), Chromosome 9 open reading frame 72 (*C9orf72*) (Renton et al., 2011), Valosin-Containing Protein (*VCP*) (Watts et al., 2004) and Charged Multivesicular Body Protein 2B (*CHMP2B*) (Skibinski et al., 2005). Moreover, there are other two subtypes of FTLD called FTLD-TDP43, characterized by tau-negative inclusions containing aggregates of TDP-43 (TAR DNA binding protein 43) (Neumann et al., 2006), and FTLD-FUS, showing inclusions of FUS (Fused in Sarcoma) protein co-localized with ubiquitin-immunoreactive inclusions (Neumann et al., 2009).

Parkinson's disease (PD) is the second most common neurodegenerative disorder leading to loss of dopamine-producing brain cells and a progressive deterioration of motor function. It is characterized by symptoms such as tremor, stiffness, slowness, impaired balance, anxiety, depression, and dementia. With regard to PD, late-onset forms of disease show mutations in α-Synuclein (*SNCA*) (Polymeropoulos et al., 1996, 1997) and Leucine-Rich Repeat Kinase2 (*LRRK2*) (Funayama et al., 2002) genes, whereas early-onset forms of disease show Parkin (*PARK2*) (Kitada et al., 1998), PTEN Induced Putative Kinase1 (*PINK1*) (Valente et al., 2001), oncogene DJ1 (*DJ1*) (Bonifati et al., 2003; Van Duijn et al., 2001) mutations.

Huntington disease (HD) is an autosomal dominant disorder associated with degeneration of the striatum. It is a genetic, progressive disorder characterized by the gradual development of involuntary muscle movements (chorea) and deterioration of cognitive processes and memory (dementia). Mutations in the Huntingtin (*HTT*) gene cause HD (MacDonald, 1993).

Amyotrophic Lateral Sclerosis (ALS) is a progressive degenerative disorder affecting upper and lower motor neurons. Loss of motor neurons results in progressive loss of voluntary muscle movement, which in turn leads to muscle atrophy. Motor impairment may eventually affect respiratory systems but cognitive functions usually remain intact. ALS is caused by different mutations, such as mutations in Superoxide Dismutase 1 (*SOD1*) (Rosen et al., 1993), in the genes coding for TDP-43 (*TARDBP*) (Sreedharan et al., 2008), *FUS/TLS*

(Sapp et al., 2003) and Ubiquilin2 (*UBQLN2*) (Kaye and Shows, 2000). Moreover, mutations in *CHMP2B* (Parkinson et al., 2006), *VCP* (Johnson J.O. et al., 2010), and *C9ORF72* (DeJesus-Hernandez et al., 2011; Renton et al., 2011) have also been implicated in ALS.

Although these are distinct pathologies in which neurodegeneration predominantly affects specific neuronal populations (dopaminergic neurons in PD, striatal medium spiny neurons in HD, motor neurons in ALS, and cortical and hippocampal neurons in AD), there are converging lines of investigation showing a clinical, pathological and genetic overlap. Some examples are the aggregation and deposition of misfolded proteins, dysfunction of RNA metabolism and processing and protein homeostasis.

The accumulation of insoluble aggregates and deposition of misfolded proteins vary from disease to disease, such as amyloid plaques mainly constituted by β-Amyloid protein (Aβ) (Glenner and Wong, 1984) and neurofibrillary tangles composed of aggregates of hyperphosphorylated Tau protein (Grundke-Iqbal et al., 1986) in AD, Lewy bodies formed by α-Synuclein (Spillantini et al., 1997) in PD, intranuclear deposits of a polyglutamine-rich version of Huntingtin protein (DiFiglia et al., 1997) in HD, aggregates mainly composed of Superoxide Dismutase (Bruijn et al., 1998) in ALS.

RNA processing is a very important step in cellular physiology in general, and in neuronal function in particular. RNA binding proteins (RBPs) and miRNAs, acting on mRNAs, add a level of gene expression regulation in the cell. Dysregulation of RBPs and miRNAs in neurons can affect RNA metabolism and, as a consequence, can lead to neuronal dysfunction and neurodegeneration.

One example of this complex regulation network comes from mislocalization of TDP-43 and FUS/TLS, observed in a large number of disorders (mainly in ALS and FTLD, but also in AD, HD and PD). All together these specific kinds of neurodegenerative diseases are called TDP-43 and FUS/TLS proteinopathies. TDP-43 and FUS/TLS are both structurally close to the family of heterogeneous ribonucleoproteins (hnRNPs) and act as regulators of multiple levels of RNA processing including transcription, splicing, transport, translation, nucleo-cytoplasmic shuttling, formation of stress granules and miRNA processing.

TDP-43 inclusions are found in ALS patients with TARDBP mutation but not in forms caused by *SOD1* mutations (Mackenzie et al., 2007), in sporadic FTLD as well as familial FTLD cases with mutations in *GRN* gene, in *VCP* gene and in rare cases with *TARDP* mutations (Cairns et al., 2007; Neumann et al., 2007). TDP-43 inclusions have also been reported in Alzheimer

(Uryu et al., 2008) and Parkinson patients (Hasegawa et al., 2007) and, in some instances, these inclusions co-exist with Tau or α-Synuclein aggregates.

FUS/TLS inclusions are less well defined but they were found in ALS and FTLD patients and in association with another kind of inclusions found in postmortem analysis of patients with different polyQ diseases (such as HD) (Doi et al., 2008).

Another example of defects in RNA metabolism comes from hexanucleotide (GGGCCC) expansion in *C9ORF72* gene (between alternative exons 1a and 1b) accounting for some ALS and FTD patients. There are at least three mechanisms of action of expanded hexanucleotide repeat in the C9ORF72 gene: haploinsufficiency due to reduced expression of the allele containing the repeat expansion; RNA toxicity due to the production of a mutant RNA containing the repeat that may sequester RNA binding proteins; protein toxicity due to the expression of a mutant protein containing the repeat expansion (La Spada and Taylor, 2010).

Other factors implicated in neurodegeneration are mitochondrial dysfunctions (shape, size, fission-fusion, distribution, movement), oxidative stress, and/or environmental factors.

Oxidative stress can be caused by an imbalance in the redox state of the cell, or by overproduction of reactive oxygen species, or by dysfunction of the antioxidant systems. A link has been demonstrated also between several environmental factors strongly associated with age, including pesticides, metals, head injuries, lifestyles and dietary habits and an increased disease risk.

Overall, these findings show that several common multifactorial processes contributing to neuronal death and leading to functional impairments are shared by many neurodegenerative diseases, making a diagnosis extremely complex.

Neurons of the central nervous system cannot regenerate after cell death and, as a consequence, once a neurodegenerative disease has manifested, significant neuronal loss and damage are already present. Therefore, it is clear the importance to establish an early diagnosis to maximize the effectiveness of disease-modifying therapies. In recent years large efforts have been made to identify disease neuropathological, biochemical, and genetic biomarkers to try to establish a diagnosis in the earlier stages. The finding that some related neurodegenerative diseases share common mechanisms suggests that these disorders may have similar targets for the development of diagnostic and therapeutic agents. Moreover the fact that the current diagnosis is based on the patient's cognitive functions increases the

need to investigate for common or similar non-invasive diagnostic methods, able to identify the disease at an early stage. Robust biomarkers would be valuable not only for the initial diagnosis, but also to classify various subtypes of the disease, to monitor responses to therapeutic agents and to track disease progression.

2. Biomarkers in Neurodegenerative diseases

Neurodegenerative disorders are different nervous system diseases characterized by the progressive loss of neuronal cells and tissues, without a possible regeneration process after the damage occurs (Rachakonda et al., 2004). The group is formed by hundreds of neurological diseases that show different symptoms, such as cognitive dysfunction or altered behavior (Krystal et al., 2014). The standard methodologies failed in the attempt to clarify and investigate on the pathological causes, due to the complex nature of neurodegenerative diseases that involves many different pathway and targets (Han et al., 2014). In the recent years there was an increasing application of sequencing and genomic approaches for the study of neurodegenerative disorders (McCarroll et al., 2014) that lead to the discovery of novel risk genes and peripheral biomarkers useful for the investigation disease mechanisms. Although genetic studies represent a key method to identify genetic risk factors through DNA sequencing and investigate the heritability process of a disease state, their function of risk assessment is clearly different from biomarkers roles (Gonzalez-Cuyar et al., 2011). Biomarkers are biological substances measured *in vivo* and used to indicate the onset or the presence of a specific disease (Rachakonda et al., 2004). The hypothetical biomarker changes are thought to begin 10-20 years before the clinical onset of a disease event. However, this timing could be influenced by the type of disorder considered, the analysis applied and the age of the subjects (Langbaum et al., 2013; Knickmeyer et al., 2014). According to this perspective the possibility to identify disease specific biomarkers at an early stage would lead to an early and maybe more effective treatment for the patients. Moreover, specific and reliable biomarkers could be applied to follow and quantify disease progression and investigate on the possible reactions to treatments, in order to achieve a better therapy (Rachakonda et al., 2004; Gonzalez-Cuyar et al., 2011). Biomarkers could also help in specific diagnosis, in discriminating similar diseases or in identifying combinations of diseases, which is enormously important for the

assembling of subjects for clinical trials (Gonzalez-Cuyar et al., 2011). Body fluids, such as plasma, serum, urine and cerebrospinal fluid (CSF) represent a useful reservoir of information of what is happening in the body. In particular CSF that is closely linked with the brain, could reflect neuro-pathological features of brain disorders (Ghidoni et al., 2011). Biomarkers that can be measured in different biological fluids can fall in two categories: proteins or microRNAs. Compared to proteins, microRNAs show many advantages such as a tissue- or cell type- specific expression, lower cost and shorter time required for the development of an assays and the presence of an amplifiable signal (Chevillet et al., 2014).

3. miRNA biogenesis and function

microRNAs (miRNAs) are a group of small non-coding RNAs with important regulatory roles on the post-transcriptional expression of target mRNAs (Bartel, 2009; Ghildiyal and Zamore, 2009), found in animals, plants, green algae and viruses (Griffiths-Jones et al., 2008). Specifically miRNAs are 21-22 nt-long single-stranded RNA molecules, firstly discovered by Lee et al. (1993), generating from longer transcripts of different lengths (pri-miRNA), usually transcribed by RNA polymerase II, from intragenic or intergenic DNA regions (Lee et al., 2004; Garzon et al., 2010). microRNAs can perform their function when they are loaded on the RNA-Induced Silencing Complex (RISC) and associates with Argonaute-2 (Ago2; Meister et al., 2004 a and b). The miRNA-RISC complex interacts with the target mRNAs, through a binding between the seed region of the miRNA, localized on its 5' end between nucleotides 2 to 8 (Bartel, 2009). However a significant fraction of non-canonical interactions, that involve non-seed base pairing can occur between the miRNA-RISC complex and the target mRNA (Helwak et al., 2013). The RISC complex induces mRNA degradation if there is a perfect complementarity between the sequences of the miRNA and its target mRNA, while the interaction leads to translation inhibition in case of imperfect binding (Bartel, 2009). Frequently miRNAs recognized bind to sequences in the 3'untranslated region (UTR) of target mRNAs, but also the coding region and the 5'UTR of a mRNA could be involved in the interaction with miRNAs (Rigoutsos, 2009; Ørom et al., 2008).

miRNAs modulate through their binding the transcriptome of cells (Guo et al., 2010). This process of post-transcriptional regulation seem to be complex and difficult to be fully

understood, since one single miRNA could potentially target hundreds of different mRNAs and one single mRNA could be controlled by many different miRNAs. Moreover miRNAs themselves can be regulated post-transcriptionally as suggested by Farajollahi and Maas (Farajollahi and Maas, 2010), creating an increasingly intricate network of regulation.

The assumption that miRNAs dysregulation has the potential to lead to neurodegeneration derived from different experiments in which knockout of Dicer, with consequent disruption of miRNAs biogenesis causes neurodegenerative phenotypes. Specifically, deletion of Dicer was performed in mouse cerebellar neurons (Schaefer et al., 2007), in midbrain dopamine neurons (Kim et al., 2007), in striatal, retinal, spinal and cortical neurons (Cuellar et al., 2008; Damiani et al., 2008; Davis et al., 2008; Haramati et al., 2010) and in glial cells (Tao et al., 2011; Shin et al., 2009; Wu et al., 2012). Several studies showed that the DGCR8 haploinsufficiency leads to a decreased production of miRNAs, with neuronal alteration as result (Stark et al., 2008; Fénelon et al., 2011; Schofield et al., 2011). Hébert and colleagues found an altered phosphorylation pattern of tau after the deletion of Dicer, suggesting the presence of miRNAs' control on specific aspects of the neurodegeneration process (Hébert et al., 2010).

Microarray analyses demonstrated a specific brain expression of different miRNAs (Lim et al., 2005; Manakov et al., 2009), which is particularly relevant during brain development (Miska et al., 2004; Kapsimali et al., 2007), whereas sequencing data lead to the development of a mammalian miRNAs expression atlas in different cell types (Landgraf et al. 2007). Recent studies showed an interesting correlation between the expression pattern of miRNAs in brains of different primates and human development (Somel et al., 2010 and 2011; Hu et al., 2011) suggesting that changes in miRNAs profiles induced significant differences at mRNA and protein levels between human and other primates' brain. Therefore, not only the global loss of miRNAs can cause neurodegeneration, but in some cases a specific alteration of a single miRNA pattern in the brain could be linked to a particular disease.

4. Circulating miRNA

RNA was considered for a long time to be unstable in the blood, due to the nuclease activity observed in human plasma (Kamm et al., 1972). However this concept changed rapidly with the observation of circulating and stable cell-free miRNAs in healthy individuals and different types of cancer patients (Mitchell et al., 2008; Asaga et al., 2011; Hu et al., 2010; Schwarzenbach et al., 2011). Subsequently many circulating miRNAs that can be used as possible biomarkers were also described for different neurodegenerative disorders (Table 1-3). However, it is not clear yet if all the reported miRNAs are directly released from cells linked with the disease or a product of aspecific secondary response.

The observation of the rapid degradation of purified or synthetic miRNAs compared with the ones found in the plasma (Mitchell et al., 2008) lead to the hypothesis of a packaging system to protect them from RNase degradation. Exosomes, which are membrane vesicles of 50-100 nm diameter found in many body fluids (Weber et al., 2010), were shown to contain proteins, mRNAs and miRNAs derived from the originating cell (Valadi et al., 2007). Moreover, microvesicles (secreted vesiscles of 1-10 μm diameter), were found to contain miRNAs associated with invasion and migration in prostate cancer (Morello et al., 2013). Several studies observed *in vitro* the cellular uptake of exosomes and other vesicles, indicating the miRNAs-containing exosomes as a possible way of communication between cells (Skog et al., 2008; Stoorvogel, 2012; Montecalvo et al., 2012; Kharaziha et al., 2012). In case of RNA transfer, the target cells' gene expression and protein translation would be modified, as a result (Pant et al., 2012). The origin of extracellular vesicles from multivesicular bodies indicates that they could have an important role for the clearance of toxins or altered proteins by the lysosomal pathway (Candelario and Steindler, 2014). Many neurodegenerative disorders, such as AD and PD, present lysosomal dysfunctions. Moreover, different proteins that lead to aggregation and accumulation in neurodegenerative disorders have been found secreted through extracellular vesiscles, such as TDP-43 (Nonaka et al., 2013), Aβ (Rajendran et al., 2006), tau (Saman et al., 2012) and α-synuclein (Emmanouilidou et al., 2010). Different studies found a large part of miRNAs which is not associated with lipid vesicles but can be affected by protease digestion (Arroyo et al., 2011; Turchinovich et al., 2011). Therefore miRNAs could circulate in biofluids also in association with different proteins, such as Ago2 (Arroyo et al., 2011) or nucleophosmin (Wang K et al., 2010) and the high-density lipoprotein (Vickers et al., 2011).

Different methods are used for the isolation, quantification and profiling of miRNAs. Total RNA extraction and isolation, guaranteeing recovery of miRNAs, is possible through commercially available column filtration protocols, or by using 'Tri-reagents' (acid phenol in combination with guanidinium-thiocyanate and chloroform). miRNAs quantification and profiling is obtained with different techniques: Next Generation Sequencing (NGS), Microarray and Real-Time PCR. Next generation sequencing (NGS) is rapidly evolving for its multiplexing capacities and provides accurate and sensitive miRNAs measurements. NGS gives the possibility to discover novel miRNAs, in contrast to microarray and qRT-PCR methods, detecting only already known miRNAs. However, NGS is labor-consuming in sample preparation and data analysis and very expensive. Another technique used for miRNA profiling is represented by microarray, showing often problems of cross-hybridisation between members of miRNA families and discrepancies in comparing results obtained with different microarray platforms. A common strategy is to validate the microarray data by qRT-PCR, to warrantee high sensitivity and specificity. Moreover, multiwell plate-based qRT-PCR assays could substitute microarrays in the high-throughput profiling of miRNAs. qRT-PCR is presently the most easily performed and cost-effective method when there is a need to measure the levels of a restricted number of miRNA as biomarkers. An important step during qRT-PCR analysis is the choice of a suitable normalization method, constituted by one or more stably expressed genes (called housekeeping or endogenous genes). This step is required to remove variations and increase the accuracy of miRNAs quantification. Ribosomal RNAs (rRNAs), U6 snRNA or a combination of different miRNAs have been used as reference genes in miRNA profiling. Finally, two approaches are frequently used for the selection of promising circulating miRNA as disease biomarkers. The first is based on an initial screening of miRNAs with subsequent validation of potential biomarkers by qRT-PCR. This kind of approach gives the possibility to study a new mechanism not previously associated with the disease. The limitations are linked to sensitivity and variability depending on the platforms used to profile miRNAs. The second approach is based on the analysis of miRNAs already associated with the disease, but it has the limitations of the potential involvement of the same miRNA in non-related diseases and of a lack of correlation between the expression of the specific miRNA in the affected organ and the relative expression of the same miRNA in plasma or CSF. This latter issue is specifically important in neurodegenerative diseases, in which the isolation of the

nervous system from the rest of the body, by the blood-brain barrier, makes it difficult for the dysregulation of a miRNA in the brain to be reflected in body fluids.

4.1 Alzheimer's disease and circulating miRNAs

Alzheimer's disease (AD) is the most prevalent cause of dementia in the elderly (Blennow et al., 2006) and shows the typical accumulation of two modified proteins: the amyloid β (Aβ) peptide, derived from the amyloid precursor protein (APP), can accumulate in diffuse or neuritic plaques (O'Brien and Wong, 2011) whereas the microtubule associated protein tau accumulates in structures called neurofibrillary tangles (NFTs). The accumulation of these proteins leads to toxic effects and inflammatory responses, contributing to the disruption of the neuronal network important for the cognitive functions (Gascon and Gao, 2012).

Dosage of proteins associated with AD in CSF is already reported as a neurochemical way for the diagnosis of AD (Blennow et al., 2009; Marksteiner et al., 2007). Specifically one of the most common peptides derived from the cleavage of APP, the 42-amminoacids-long $A\beta_{42}$, was found decreased in CSF of AD patients (Galasko et al., 2010). Similarly, elevated levels of total and phosphorylated tau in the CSF are AD biomarkers (Blennow et al., 2009; Marksteiner et al., 2007; Fagan et al., 2007). Regarding the altered expression of miRNAs in AD tissues and their pathogenic role, a study identified miR-107 to be decreased during AD (Wang W.X. et al., 2008), through a miRNA expression profiling in cortex from control and AD patients. The mRNA of BACE1, the protease involved in the cleavage of APP was predicted as target of miR-107 and the miRNA's role was confirmed by expression studies. mRNA profiling showed that BACE1 mRNA levels tented to increase as miR-107 decreased during the progression of AD, indicating how this microRNA could be involved in the pathological process of Alzheimer's disease (Wang W.X. et al., 2008). Regulation and control of BACE1 by another cluster of miRNAs (miR-29a/b-1) was further investigated by Hébert et al. (Hébert et al., 2008). Moreover, a study demonstrated up-regulated levels of miR-9 and miR-128 in AD hippocampus (Lukiw et al., 2007) and another work found increased levels of miR-9, miR-125b and miR-146a in the temporal lobe neocortex of affected AD individuals (Sethi et al., 2009), suggesting that miRNAs pattern can be altered in sporadic AD.

The recent research demonstrated that miRNAs are not only aberrantly expressed in AD or involved in the regulation of the main pathological processes, but also that their altered presence in different body fluids could be diagnostic of AD (Table 1). The first study that investigated peripheral miRNAs expression in Alzheimer condition was performed in 2007 (Schipper et al., 2007). The authors identified an increased level of miRNAs in blood mononuclear cells (BMC) of patients with sporadic AD using a microarray containing 462 human miRNAs. In particular miR-34a and miR-181b were significantly increased in AD individuals, as validated through qPCR (Schipper et al., 2007). In 2012 a decreased level of some circulating miRs measured through SYBR Green qRT-PCR in the blood serum of AD patients was suggested as a non-invasive diagnostic method for Alzheimer's disease (Geekiyanage et al., 2012). Around the same year another work used plasma miRNAs quantified through TaqMan miRNA qRT-PCR assay to detect mild cognitive impairment (MCI), that represents an intermediate state between normal aging and AD or other types of dementia (Sheinerman et al., 2012). This group demonstrated that ratio of plasma levels of miR-132 and miR-134 families paired with miR-491-5p and miR-370 differentiate MCI subjects from controls, with high sensitivity and specificity (Sheinerman et al., 2012). In particular they identified two sets of paired biomarkers ratio: the miR-132 family (miR-128/miR-491-5p, miR-132/miR-491-5p and miR-874/miR-491-5p) and the miR-134 family (miR-134/miR-370, miR-323-3p/miR-370 and miR-382/miR-370). Subsequently Leidinger and co-workers identified in the blood of AD patients a signature of 12 miRNAs that can be useful for the diagnosis. They applied next-generation sequencing to select a panel of 12 miRNAs that were further used through RT-qPCR in larger cohort of samples, differentiating with high accuracy and specificity between AD and controls or between AD patients and individuals suffering of other neurological diseases (Leidinger et al., 2013). The signature contains 7 up-regulated miRNAs in AD (brain-miR-112, brain-miR-161, hsa-let-7d-3p, hsa-miR-5010-3p, hsa-miR-26a-5p, hsa-miR-1285-5p and hsa-miR-151a-3p) and 5 down-regulated miRNAs in AD patients (hsa-miR-103a, hsa-miR-107, hsa-miR532-5p, hsa-miR-26b-5p and hsa-miR-let-7f-5p) (Leidinger et al., 2013). A different signature of 7 miRNAs (miR-let-7d-5p, miR-let-7g-5p, miR-15b-5p, miR-142-3p, miR-191-5p, miR-301a-3p and miR-545-3p) was identified in the plasma to distinguish AD patients from normal subjects with a good accuracy (Kumar et al., 2013). Another work that evaluated the possibility to use miRNAs expressed in human brain and biofluids as biomarkers of AD, was performed by Bekris and

collaborators, in which miR-15a level was shown to positively correlate with neuritic plaque score (Bekris et al., 2013). In particular a higher plasma miR-15a level was suggested to be a feasible marker of high neuritic plaque in hippocampus (Bekris et al., 2013). Bhatnagar and collaborators studied through TaqMan array and validated with qRT-PCR, miRNAs circulating in plasma and blood mononuclear cells (PBMC) of AD patients, identifying miR-34c as a biomarker to individuate sporadic AD subjects, since its level is significantly increased in plasma samples of patients (Bhatnagar et al., 2014). During the last year one work showed an altered expression of 6 miRNAs (miR-98-5p, miR-885-5p, miR-483-3p, miR-342-3p, miR-191-5p, miR-let-7d-5p) in serum of AD patients compare to controls, with high specificity and sensitivity for miR-342-3p (Tan et al. 2014). More recently Galimberti and colleagues correlated altered miRNAs expression between serum and CSF of AD patients campare to healthy individuals, finding downregulation of 3 miRNAs (miR-125b, miR-23a and miR-26b) in serum derived from 22 AD patients. Downregulation of miR-125b and miR-26b was also observed and confirmed in CSF from AD individuals (Galimberti et al. 2014). The function of miR-125b was also recently validated in vivo, indeed injection of miR-125b into hippocampus in mice induces tau phosphorylation and learning impairment (Banzhaf-Strathmann et al. 2014).

The first time in which CSF was used to identify possible biomarkers for the diagnosis of AD was in the 2008, Cogswell and coworkers discovered that miRNAs can be detected in this fluid and their expression is altered in the presence of AD pathology. In particular they found altered miRNAs related to immune cell differentiation and innate immunity (Cogswell et al., 2008). Significant increases in the levels of miR-9, miR-125b, miR-146a and miR-155 was found in CSF and in extracellular fluid (ECF) derived from short post-mortem interval brain tissue of AD patients (Alexandrov et al., 2012). Some of these miRNAs are known to be over-express in brain of patients and associated with the spreading of inflammatory neurodegeneration (Alexandrov et al., 2012). Another candidate biomarker for AD is miR-27a-3p, which was found reduced in the CSF of patients compare to control subjects in a pilot study (Sala Frigerio et al., 2013). The decrease in the level of miR-27a-3p is combined with high CSF tau levels and low CSF β-amyloid levels (Sala Frigerio et al., 2013).

As already reported (Cogswell et al., 2008) CSF- and ECF-derived from AD patients contain abundant levels of proinflammatory miR, in particular miR-146a and miR-155 (Lukiw et al., 2012). These two miRNAs were suggested to be involved in the spreading process of

Alzheimer's disease, since human neuronal-glial cocultures secrete these miRNAs upon cytokine tumor necrosis factor-α (TNF-α) and Aβ42-peptide stress; in addition it was observed that a conditioned medium containing miR-146a and miR-155 induces inflammatory gene expression and downregulation of complement factor H (CFH). This regulator is involved in inflammatory degeneration in Alzheimer's disease and other disorders (Lukiw et al., 2012). A similar function was proposed for miR-let-7, that was found up-regulated in the CSF of AD subjects. They demonstrated that extracellular introduction of let-7b in the CSF of mice induces neurodegeneration through the RNA-sensing Toll-like receptor (TLR) 7 (Lehmann et al., 2012). miR-146a was also identified in plasma and CSF of AD patients in another recently study with other candidates miRNAs (Kiko et al., 2014). However its level was analysed through qRT-PCR and found significantly dowregulated in AD patients compared to controls in apparently contrast with previous reports (Alexabdrov et al., 2012; Lukiw et al. 2012).

4.2 Parkinson's disease and circulating miRNAs

Approximately 1% of the population over the age of 55 is affected by Parkinson's disease (PD). This neurodegenerative disorder is characterized by the degeneration of dopaminergic neurons of the substantia nigra that leads to rigidity, tremors and slowed movements. Another pathological feature is the presence of inclusions primarily composed of α-synuclein, called Lewy bodies. These inclusions are cytoplasmic and show a characteristic pattern in the brain. One of the biochemical markers used to recognize the onset of PD is the loss of the dopamine transporter (DAT) or the identification of α-synuclein protein in the Lewy bodies (Duyckaerts et al., 2003). Ruling out the analysis of protein involved in the pathology in the brain, an increased level of oxidative stress markers in blood of PD patients, such as superoxide radicals and the coenzyme Q10 redox ratio were suggested as potential biomarker for Parkinson's disease (Michell et al., 2004). It was also shown that the proinflammatory factor tumor necrosis factor (TNF-α) is 3-4 fold higher in CSF of PD patients compared to controls (Le et al., 1999). Decreased levels of α-synuclein concentration in CSF of PD patients have been found by different laboratories (Mollenhauer et al., 2011; Shi et al., 2011). In addition some groups investigated as biomarker of PD, the CSF level of a multifunctional redox-sensitive protein, important for mitochondrial function, called DJ-1

(Waragai et al., 2006; Hong et al., 2010). However the studies highlighted the necessity of controlling blood contamination of CSF, age during the analysis (Hong et al. 2010) and the clear need of an improved laboratory test with higher performance (Shi et al. 2011). So far none of these potential biomarker seems to be sufficiently robust and specific to be useful as a real diagnostic biomarker in clinical practice.

Regarding the presence of circulating miRNAs in the body fluids of PD patients (Table 2), the first study that investigate blood samples was performed by Margis and collaborators in the 2011 (Margis et al., 2011). They found through qRT-PCR analysis three differential expressed miRNAs: miR-1, miR-22-5p and miR-29 that could be used to distinguish between not-treated PD patients and normal controls individuals; whereas miR-16-2-3p, miR-26a-2-3p and miR-30a are differentially expressed between treated and untreated PD patients (Margis et al., 2011). In the same year another study found using miRCURY LNA microarrays, 18 miRNAs with an altered expression in the peripheral blood mononuclear cells (PBMCs) of PD subjects, and predicted that target genes of these miRNAs were involved in PD's pathological pathways (Martins et al. 2011). More recently a study identified through RNA-Seq, 16 miRNAs differentially expressed in blood leukocytes of PD patients compared to healthy control volunteers (Soreq et al., 2013). Eleven miRNAs were modified after deep-brain stimulation (DBS) treatment, whereas five were changed inversely to the disease-induced changes (Soreq et al., 2013). Investigation and analysis of miRNAs expression in plasma of PD patients was also performed by Khoo and coworkers (Khoo et al., 2012). Using microarrays and TaqMan qRT-PCR validation, they identified four miRNAs: miR-1826, miR-450b-3p, miR626 and miR-505, whose levels can be used in combination to obtain the highest predictive biomarker performance to individuate the presence of the disorder (Khoo et al., 2012). Another work that analyzed plasma from PD patients and normal controls through TaqMan miRNA qRT-PCR, identified miR-331-5p as a possible PD biomarker (Cardo et al., 2013). Recently a study investigated circulating miRNAs in serum of PD or multiple system atrophy (MSA) patients compared to healthy controls to distinguish individuals that are affected by these two different pathologies with overlapping features (Vallelunga et al., 2014). Specifically, through array analysis and qRT-PCR validation, the authors identified 4 miRNAs that are downregulated and five miRNAs that are upregulated in PD serum versus

control subjects (Vallelunga et al., 2014). miRNAs profiling of serum derived from idiopathic Parkinson's disease (IPD), PD patients carriers the LRRK2 G2019S mutation and controls was also performed through real time PCR- based TaqMan MicroRNA arrays, finding downregulation of miR-29a, miR-29c, miR-19a and miR-19b in patients compare to healthy individuals (Botta-Orfila et al. 2014). So far there are no studies reporting miRNA biomarkers for PD in patients' CSF.

4.3 Amyotrophic lateral sclerosis and circulating miRNAs

As already mentioned, ALS is caused by mutations in SOD1, in RNA-binding proteins as TDP-43 and FUS/TLS, in CHMP2B, VCP, and C9ORF72. Some miRNAs are emerging as important contributors to ALS pathogenesis.

The involvement of TDP-43 and FUS/TLS in miRNAs biogenesis, has been uncovered, as these proteins directly bind key components of the miRNA processing pathway. Drosha is able to form two distinct protein complexes, a "more canonical" complex with DGCR8 and a larger complex, including TDP-43 and FUS, with limited pri-miRNA processing activity (Gregory et al., 2004). In addition, TDP-43 was shown to directly bind Dicer, Ago2, subsets of pri-miRNAs in the nucleus and pre-miRNAs in the cytoplasm (Kawahara and Mieda-Sato, 2012). *In vitro* depletion of TDP-43 and FUS proteins leads to a reduction of specific subsets of miRNAs implicated in neuromuscular development, neuronal function and survival (Buratti et al., 2010; Kawahara and Mieda-Sato, 2012; Morlando et al., 2012).

To determine if miRNAs are essential to motor neuron survival, Haramati's group in 2010 used Dicer knockdown mice. They demonstrated that the heavy neurofilament subunit is a target of miR-9, already reported to be down-regulated in a genetic model of Spinal Muscular Atrophy (SMA) (Haramati et al., 2010). It has been shown that SMA and ALS are motor neuron diseases linked by a common molecular pathway: FUS, mutated in ALS, interacts with SMN, deficient in SMA (Yamazaki et al., 2012). Moreover, in a recent study, a miR-9 reduction was found in human neurons derived from Induced Pluripotent Stem Cells (iPSCs) from patients with the pathogenic TARDBP M337V mutation, suggesting that miR-9 down-regulation could be a common pathogenic event in FTD/ALS (Zhang et al., 2013). In a recent study alteration in ALS of some miRNAs directly targeting neurofilament light chain

mRNA (NEFL) has been shown. Among these dys-regulated miRNAs, there are miR-146a* (up-regulated) and miR-524-5p and miR-582-3p (down-regulated) in spinal cord (SC) from sporadic ALS (sALS) patients (Campos-Melo et al., 2013). In addition, a group of 80 putative novel miRNAs from control and sporadic ALS (sALS) spinal cords has been characterized. Among them, 24 have miRNA response elements (MREs) within the NEFL mRNA 3'UTR and 2 of them, miR-b1336 and miR-b2403, are down-regulated in ALS spinal cord (Ishtiaq et al., 2014).

Changes in miRNAs have also been seen in peripheral ALS tissues. The muscle-specific miR-206 is up-regulated in lower limbs of SOD1-G93A mice (Williams et al., 2009) and in a mouse model with miR-206 deletion, acceleration of disease progression was observed, suggesting that the high amount of miR-206 in SOD1-G93A mice may have a compensatory effect to reduce degeneration in ALS. A similar increase in miR-206 was also observed in ALS patients' muscle tissue (Russell et al., 2012). In the same work the authors found that skeletal muscle mitochondrial dysfunction in ALS patients is associated with an increase in some miRNAs (miR-23a, 29b, 206 and 455) and with a reduction in peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) signaling pathways (Russell et al., 2012).

In another work, high expression of miR-29a was observed in brain and spinal cord of SOD1 (G93A) mice, a model for familial ALS. These results provide a first evidence for the possible therapeutic utility of modulation of miR-29a function in ALS (Nolan et al., 2014). In a very recent study, miR-141 and miR-200a are found to be linked with FUS by a feed-forward regulatory in which FUS up-regulates miR-141/200a, which in turn regulate FUS protein synthesis. Moreover, Zeb1, a miR-141/200a target and at the same time a transcriptional repressor of these two miRNAs, is part of the circuitry and reinforces it (Dini Modigliani et al., 2014).

A small number of studies recently investigated miRNAs as ALS biomarkers in CSF and in blood (Table 3). A first study was performed on leukocytes from sALS patients with respect to healthy controls. The study reported a profile of 8 miRNAs significantly up- or down-regulated in sALS patients (De Felice et al., 2012). Another study performed the analysis on sorted CD14+ CD16– monocytes from ALS patients, which are monocytes activated and

recruited to the spinal cord in case of inflammation correlated with neuronal loss. This study showed a miRNAs profile constituting an inflammatory signature that could be useful as biomarker for disease stage or progression (Butovsky et al., 2012). The populations of deregulated miRNAs found in leucocytes (De Felice et al., 2012) and monocytes (Butovsky et al., 2012) are not overlapping and comparable, although the two analyses were performed with the same technical approach, TaqMan miRNA assay-based quantitative RT-PCR (qRT-PCR).

Recently, other two miRNAs emerged as possible candidates as ALS biomarkers in biofluids. In the first, miRNA alterations were studied in plasma of SOD1-G93A mice, and subsequently in the serum of human ALS patients (Toivonen et al., 2014). miR-206 was increased in plasma of symptomatic animals and in ALS patients, showing to be a promising candidate biomarker for this motor neuron disease (Toivonen et al., 2014). In the second study, up-regulation of miR-338-3p in blood leukocytes as well in cerebrospinal fluid, serum, and spinal cord from sALS patients was detected (De Felice et al., 2014).

4.4 Huntington's disease and circulating miRNAs

The causal mutation of HD is an expanded repetition of the CAG trinucleotide in the first exon of the gene encoding huntingtin (HTT). HTT associates with Ago2 in P-bodies, and HTT knockdown has an effect on gene silencing mediated by miRNAs as demonstrated by Luciferase assay in which HTT kd abrogates let-7b silencing effect (Savas et al., 2008). miRNAs were implicated in HD pathogenesis. The repressor element 1 silencing transcription (REST) factor, a transcriptional repressor acting to silence neuronal gene expression in non-neuronal cells, is elevated in HD neurons, and this up-regulation gives as result a repression of key neuronal genes (Zuccato et al., 2007; Johnson R. et al., 2010). REST and its cofactor coREST have target sites for miR-9 and miR-9*, respectively (Packer et al., 2008), and these two miRNAs, together with miR-7, miR-124, miR-132, and other miRNAs result to be down-regulated in HD patients (Johnson R. et al., 2008; Martí et al., 2010). There are also deregulated miRNAs not under REST control, suggesting that miRNA dysregulation is extensive in HD (Jin et al., 2012; Sinha et al., 2010). In cellular models of HD, miR-146a, miR-125b, and miR-150 are down-regulated in the presence of mutant HTT protein (Sinha et al., 2010). Interestingly, miR-146a, miR-150, and miR-125b target HTT and

were also predicted to interact with the TATA binding protein (TBP) mRNA. This protein is known to be recruited into mutant HTT aggregates (Sinha et al., 2010 and 2011). In the cortex of mutant HTT mouse models at early stages of disease miR-200 family is altered, compromising genes involved in neuronal plasticity and survival (Jin et al., 2012).

With regard to biofluids, it has been demonstrated that miR-34b is up-regulated in response to mutant HTT in human plasma, suggesting a possible role for miR-34b as a biomarker for HD (Gaughwin et al., 2011).

4.5 Other diseases

Some miRNAs were linked to FTD through different mechanisms. For example, miR-29b and miR-107 regulate progranulin levels (Jiao et al., 2010; Wang W.X. et al., 2010). These miRNAs might decrease progranulin levels and, since it has been demonstrated that progranulin haploinsufficiency can cause FTD, might be a risk factor for this disease. Moreover, a genetic polymorphism (rs5848) in PGRN 3'UTR, associated with a higher risk of FTD, affects miR-659 binding site resulting in a more efficient binding and, as a consequence, in decreased progranulin levels (Rademakers et al., 2008). Finally, several miRNAs were found deregulated in FTD with TDP-43 pathology (Kocerha et al., 2011). Unfortunately, no studies were performed about circulating miRNA in plasma or serum from patients with FTD, suggesting that the study of circulating miRNA in plasma/serum for diagnosis of this kind of disorder needs more detailed investigations.

5. Therapy

The advantages in the use of miRNAs are that these very small RNAs have the ideal biomarker characteristics: ease of detection, extreme specificity and remarkable stability. The use of miRNA as therapeutic agents for neurodegenerative diseases is an important opportunity but also a challenging topic for several reasons. Each miRNA can regulate numerous target of the same pathway and so they could be a promising tool in which low dose of the top miRNA would be enough to induce a focused change in the entire pathway. However, these same features also present great challenges. There are still many notions to

be elucidated, such as all the functional targets for each miRNA in specific cell types or the hierarchic order of miRNA regulation in a specific pathway and, obviously, the delivery of therapeutics miRNAs into the brain due to the blood-brain barrier.

There are different miRNA-based therapeutic strategies *in vivo*. miRNA mimics are small RNA molecules very similar to miRNA precursors and could be used to potentiate the miRNA post-transcriptional regulation role in some disease conditions in which one miRNA is down-regulated. On the other hand, it would be possible to block over-expressed miRNAs in other kind of diseases by injecting a complementary RNA sequence (anti-miRNA) that binds to and inactivates the target miRNAs. A different experimental strategy to inhibit miRNA function could be mediated by synthetic sponge mRNA, containing complementary binding sites for a miRNA of interest.

In the first strategy of miRNA-based therapeutics some challenges are its potential off-target effects and the possibility to saturate the endogenous miRNA processing machinery and to interfere with the normal functions of the cell. Another big challenge for this approach is the delivery of the miRNA to the right cells in the body. It has been shown that exosomes, endogenous nanovesicles transporting RNAs and proteins, can deliver siRNA to the brain of mice when injected intravenously (Alvarez-Erviti et al., 2011). This finding paves the way for a possible therapeutic approach mediated by exosomes opening the possibility to load miRNAs into the exosomes and to target them to the brain following systemic delivery. This approach has great potential but it will have to be explored further. In the second strategy locked nucleic acids (LNAs), a class of bicyclic conformational analogs of RNA, exhibiting high binding affinity to complementary RNA target molecules and high stability *in vivo* (Fluiter et al., 2003; Hutvágner et al., 2004) are used to enhance the specificity and to reduce the amount of anti-miRNA molecules. However, those "antagomirs" cannot cross the blood-brain barrier, but can enter into brain cells only if injected directly into the brain (Kuhn et al., 2010).

Finally, the sponge technology has some advantages in its experimental settings. For example many miRNAs have the same seed sequence but are encoded by multiple distant loci and, therefore, sponges provide a way to sequestrate at the same time different

miRNAs having the same seed sequence. However also this technology needs further investigations to be a therapeutically viable strategy.

In conclusion, despite many scientific questions still open and a need of further investigations, the analysis of miRNA as biomarkers for neurodegenerative pathologies could be helpful to develop minimally invasive screening tests and to find diagnostic, prognostic and therapeutic tool for these diseases.

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Table 1. Alzheimer's Disease and circulating miRNAs

SAMPLE	miRNA	TREND	EXPERIMENTAL APPROACH	PILOT STUDY	VALIDATION	REFERENCES
PBMC	miR-34a, miR-181b	Upregulation	miRNA profiling	Microarray AD:16 NEC:16	Taqman® miRNA qRT-PCR	Schipper et al. 2007
Blood serum	miR-137, miR-181c, miR-9, miR-29a/b	Downregulation	candidate miRNAs	SYBR qRT-PCR AD:7 MCI/Early AD:7 CT:7		Geekiyanage et al. 2012
Plasma	miR-132 family, miR-134 family, miR-491-5p, miR-370	Ratio of miRNAs paired	candidate miRNAs	Taqman® miRNA qRT-PCR MCI:10 CT:10	TaqMan® qRT-PCR MCI: 20 AD: 20 CT: 20 CY: 20	Sheinerman et al. 2012
Blood	12-miRNA signature: miR-112, miR-161, let-7d-3p, miR-5010-3p, hsa-miR-26a-5p, hsa-miR-1285-5p, and hsa-miR-151a-3p	Upregulation	miRNA profiling	Next Generation Sequencing AD: 48 CT: 22	SYBR qRT-PCR AD: 94 MCI: 18 MS: 16 PD: 9 DEP: 15 BD: 15 Schiz: 14 CT: 21	Leidinger et al. 2013
Blood	12-miRNA signature:miR-103a-3p, miR-107, miR-532-5p, miR-26b-5p, let-7f-5p	Downregulation	miRNA profiling	Next Generation Sequencing AD: 48 CT: 22	SYBR qRT-PCR AD: 94 MCI: 18 MS: 16 PD: 9 DEP: 15 BD: 15 Schiz: 14 CT: 21	Leidinger et al. 2013

Plasma	7-miRNA signature: hsa-let-7d-5p, hsa-let-7g-5p, hsa-miR-15b-5p, hsa-miR-142-3p, hsamiR-191-5p, hsa-miR-301a-3p and hsa-miR-545-3p		miRNA profiling	Nanostring AD:11 MCI:9 CT:20	Taqman® miRNA qRT-PCR AD:20 CT:17	Kumar et al. 2013
Plasma	miR-15a associated with amyloid plaque score in hippocampus region	Downregulation	Candidate miRNAs	Microarray	qRT-PCR	Bekris et al. 2013
cell free Plasma or PBMC	miR-34c	Upregulation	Candidate miRNAs	Taqman® miRNA qRT-PCR AD:110 CT:123		Bhatnagar et al. 2014
Plasma	miR-34a, miR-146a	Downregulation	Candidate miRNAs	qRT-PCR		Kiko et al. 2014
Serum	miR-98-5p, miR-885-5p, miR-483-3p, miR-342-3p, miR-191-5p, miR.191-5p, miR-let-7d-5p	Upregulation and Downregulation	miRNA profiling	Illumina HiSeq 2000 sequencing AD:50 CT:50	qRT-PCR AD:158 CT:155	Tan et al. 2014
Serum	miR-125b, miR-23a, miR-26b	Downregulation		Array AD:22 NINDCs:18 INDCs:18 FTD:10	qRT-PCR	Galimberti et al. 2014
CSF	60 miRNAs differently express between Braak stage V and Braak	Upregulation and Downregulation	miRNA profiling	Taqman® miRNA qRT-PCR Braak stage V AD:10 Braak stage I		Cogswell et al. 2008

	stage I			CT:10		
CSF ECF	miRNA-9, miRNA-125b, miR-146a, miR-155	Upregulation	candidate miRNAs	Microarray AD:5 CT:5	LED-Northern dot-blot assay using primary human neuronal-glial cell-cultures and AD ECF	Alexandrov et al. 2012
CSF	miR-27a-3p	Downregulation	miRNA profiling	qRT-PCR AD CT	qRT-PCR AD:35 CT:37	Sala Frigerio et al. 2013
CSF ECF	miR-146a, miR-155	Upregulation	candidate miRNAs	Microarray AD:5 CT:5	LED-Northern dot-blot assay using primary human neuronal-glial cell-cultures and AD ECF	Lukiw et al. 2012
CSF	miR-let-7b	Upregulation	candidate miRNAs	Taqman® miRNA qRT-PCR AD:13 CT:11	mice experiments	Lehmann et al. 2012
CSF	miR-34a, miR-125b, miR-146a	Downregulation	candidate miRNAs	qRT-PCR		Kiko et al. 2014
CSF	miR-29a, miR-29b	Upregulation	candidate miRNAs	qRT-PCR		Kiko et al. 2014
CSF	miR-125b, miR-26b	Downregulation	miRNA profiling	miRNA PCR array AD:22 FTD:10 CT:18	Taqman qRT-PCR	Galimberti et al. 2014

(PBMC: Peripheral Blood Mononuclear Cells; NEC: Normal Elderly Controls; AD: Alzheimer Disease; CT: Control; NINDCs: non-inflammatory neurological controls; INDCs: inflammatory neurological controls; MCI: Mild Cognitive Impairment; MS: Multiple Sclerosis; PD: Parkinson Disease; DEP: MajorDepression; BD: Bipolar Disorder; Schiz: Schizophrenia; CSF: Cerebrospinal Fluid; ECF: brain tissue-derivedextracellular fluid; CY: Young Control; FTD: Frontotemporal Dementia)

Table 2. Parkinson's Disease and circulating miRNAs

SAMPLE	miRNA	TREND	EXPERIMENTAL APPROACH	PILOT STUDY	VALIDATION	REFERENCES
Total peripheral blood	miR-1, miR-22-5p, miR-29 distingiush non-treated PD from healthy subjects	Downregulation	miRNA profiling	qRT-PCR untreated PD: 8 treated PD: 4 early-onset PD: 7 CT:8		Margis et al. 2011
Total peripheral blood	miR-16-2-3p, miR-26a-2-3p, miR-30a distinguish treated from untreated PD	Upregulation	miRNA profiling	qRT-PCR untreated PD: 8 treated PD: 4 early-onset PD: 7 CT:8		Margis et al. 2011
PBMC	18 miRNAs	Downregulation	miRNA profiling	Microarray PD:19 CT:13		Martins et al. 2011
Leukocytes	16 miRNAs differentially expressed: 6 miRNAs	Downregulation	miRNA profiling	Next Generation Sequencing PD pre-DBS: 3 PD post-DBS: 3 CT:6		Soreq et al. 2013
Leukocytes	16 miRNAs differentially expressed: 10 miRNAs	Upregulation	miRNA profiling	Next Generation Sequencing PD pre-DBS: 3 PD post-DBS: 3 CT:6		Soreq et al. 2013
Plasma	miR-1826, miR-450b-3p, miR-626, miR-505	Upregulation of k-Top Scoring Pairs (k-TSP1) (miR-1826/miR-450b-3p), miR-626, miR505	miRNA profiling	Agilent microarray PD:32 CT:32	Taqman® miRNA qRT-PCR PD:30 MSA:4 PSP:5 CT:8	Khoo et al. 2012
Plasma	miR-331-5p	Upregulation	miRNA profiling	Taqman® miRNA qRT-PCR		Cardo et al.2013

				PD:31 CT:25		
Serum	miR-339-5p, miR-652, miR-1274A, miR-34b	Downregulation	miRNA profiling	Taqman® Human microRNA Array PD:6 MSA:9 CT:5	Taqman® miRNA qRT-PCR PD:25 MSA:25 CT:25	Vallelunga et al. 2014
Serum	miR-223*, miR-324-3p, miR-24, miR-148b, miR-30c	Upregulation	miRNA profiling	Taqman® Human microRNA Array PD:6 MSA:9 CT:5	Taqman® miRNA qRT-PCR PD:25 MSA:25 CT:25	Vallelunga et al. 2014
Serum	miR-29a, miR-29c, miR-19a, miR-19b	Downregulation	miRNA profiling	Taqman® Human microRNA Array IPD:10 PD:10 CT:10	Taqman® Human microRNA Array IPD:20 PD:20 CT:20	Botta-Orfila et al. 2014

(PD: Parkinson Disease; PSP: Progressive Supranuclear Palsy; MSA: Multiple System Atrophy; DBS: Deep Brain Stimulation; IPD: Idiopathic Parkinson's Disease).

Table 3. Amyotrophic Lateral Sclerosis and circulating miRNAs

SAMPLE	miRNA	TREND	EXP. APPROACH	PILOT STUDY	VALIDATION	REFERENCES
Leukocytes	miR-338-3p	Upregulation	miRNA profiling	Microarray ALS:8 CT:12	Taqman® miRNA qRT-PCR ALS:14 CT:14	De Felice et al., 2012
Leukocytes	miR-451, miR-1275, miR-328, miR-638, miR-149, miR-665, miR-583	Downregulation	miRNA profiling	Microarray ALS:8 CT:12	Taqman® miRNA qRT-PCR ALS:14 CT:14	De Felice et al., 2012
Monocytes	miR-27a, miR-155, miR-146a, miR-532-3p	Upregulation	miRNA profiling	Microarray ALS:8 MS:8 CT:8		Butovsky et al., 2012

(ALS: Amyotrophic lateral sclerosis)

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Title: Reduced miR-659-3p levels correlate with progranulin increase in hypoxic conditions: implications for frontotemporal dementia.

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Keywords: progranulin, miR-659-3p, frontotemporal dementia, hypoxia, asphyxia, HeLa, SK-N-BE, rats

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Abstract: Progranulin (PGRN) is a secreted protein expressed ubiquitously throughout the body, including the brain, where it localizes in neurons and activated microglia. Loss-of-function mutations in the GRN gene are an important cause of familial Frontotemporal Lobar Degeneration (FTLD). PGRN has neurotrophic and anti-inflammatory activity and it is neuroprotective in several injury conditions, such as oxygen or glucose deprivation, oxidative injury and hypoxic stress. Indeed, we have previously demonstrated that hypoxia induces the up-regulation of the GRN mRNA transcripts.
Several studies have shown the involvement of microRNAs in hypoxia. Moreover, in FTLD patients with a common genetic variant of GRN (rs5848) the reinforcement of the binding site for miR-659-3p has been suggested to be a risk factor. Here, we report that miR-659-3p interacts directly with the GRN 3'UTR as shown by luciferase assay and ELISA and Western Blot analysis in HeLa cells. Moreover, we demonstrate the physical binding between GRN mRNA and miR-659-3p employing a miRNA capture-affinity technology in SK-N-BE cells. In order to study a possible involvement of miRNAs in hypoxia-mediated up-regulation of GRN, we evaluated miR-659-3p levels in SK-N-BE cells after 24 h of hypoxic treatment finding them inversely correlated to GRN transcripts. Furthermore, we extended our experimental work to an animal model of asphyxia finding that GRN mRNA levels increased at pnd 1 and pnd 4 in cortices of rats subjected to 20 min asphyxia in comparison to control rats and miR-659-3p decreased at pnd 4 just when GRN reached the highest levels. In conclusion, our results demonstrate the interaction between miR-659-3p and GRN transcript and the involvement of miR-659-3p in GRN up-regulation mediated by hypoxic/ischemic insults.

*Cover Letter

April 22nd 2015

To Dr. O. Isacson

Editor - in - Chief, Molecular and Cellular Neuroscience
Ctr. for Neuroregeneration Research, Harvard Medical School, 115 Mill Street, Belmont, MA 02478, Massachusetts, USA, Fax: +1 617 855 3284

Sir,

We would like to submit to Molecular and Cellular Neuroscience the manuscript entitled "Reduced miR-659-3p levels correlate with progranulin increase in hypoxic conditions: implications for frontotemporal dementia" edited by Paola Piscopo, Margherita Grasso, Francesca Fontana, Alessio Crestini, Maria Puopolo, Valerio Del Vescovo, Aldina Venerosi, Gemma Calamandrei, Sebastian F. Vencken, Catherine M. Greene, Annamaria Confaloni and Michela A. Denti for publication as Regular article.

I state that all authors have contributed to the work, agree with the presented findings, and that the work has not been published before nor is being considered for publication in another journal.

I state that procedures involving animal experiments were carried out in accordance with The EU Directive 2010/63/EU.

I hope that our paper will be of interest for your readers and acceptable for publication in your Journal.

Thank you for your consideration.

Looking forward to hearing from you.

Sincerely yours

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Reduced miR-659-3p levels correlate with progranulin increase in hypoxic conditions: implications for frontotemporal dementia.

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Keywords: progranulin, miR-659-3p, frontotemporal dementia, hypoxia, asphyxia, HeLa, SK-N-BE, rats

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Abstract

Progranulin (PGRN) is a secreted protein expressed ubiquitously throughout the body, including the brain, where it localizes in neurons and activated microglia. Loss-of-function mutations in the *GRN* gene are an important cause of familial Frontotemporal Lobar Degeneration (FTLD). PGRN has neurotrophic and anti-inflammatory activity and it is neuroprotective in several injury conditions, such as oxygen or glucose deprivation, oxidative injury and hypoxic stress. Indeed, we have previously demonstrated that hypoxia induces the up-regulation of the *GRN* mRNA transcripts.

Several studies have shown the involvement of microRNAs in hypoxia. Moreover, in FTLD patients with a common genetic variant of *GRN* (rs5848) the reinforcement of the binding site for miR-659-3p has been suggested to be a risk factor. Here, we report that miR-659-3p interacts directly with the *GRN* 3'UTR as shown by luciferase assay and ELISA and Western Blot analysis in HeLa cells. Moreover, we demonstrate the physical binding between *GRN* mRNA and miR-659-3p employing a miRNA capture-affinity technology in SK-N-BE cells. In order to study a possible involvement of miRNAs in hypoxia-mediated up-regulation of *GRN*, we evaluated miR-659-3p levels in SK-N-BE cells after 24 h of hypoxic treatment finding them inversely correlated to *GRN* transcripts. Furthermore, we extended our experimental work to an animal model of asphyxia finding that *GRN* mRNA levels increased at pnd 1 and pnd 4 in cortices of rats subjected to 20 min asphyxia in comparison to control rats and miR-659-3p decreased at pnd 4 just when *GRN* reached the highest levels. In conclusion, our results demonstrate the interaction between miR-659-3p and *GRN* transcript and the involvement of miR-659-3p in *GRN* up-regulation mediated by hypoxic/ischemic insults.

1. Introduction

1.1 Progranulin and hypoxia

Progranulin (PGRN) is a 65 kDa secreted protein expressed ubiquitously throughout the body, including the brain, where it localizes in neurons and activated microglia (Daniel et al., 2003; Matsuwaki et al., 2011; Petkau et al., 2010). Loss-of-function mutations in the *GRN* gene are an important cause of familial Frontotemporal Lobar Degeneration (FTLD) with TAR DNA-binding protein 43 (TDP-43)-positive inclusions (FTLD-TDP). Biological activities attributed to progranulin are numerous, yet their relevance to neurodegeneration is unclear. PGRN has neurotrophic and anti-inflammatory activity (Zhu et al., 2002; Kessenbrock et al., 2008; Tang et al., 2011; Gass et al., 2012; De Muynck et al., 2013) and is neuroprotective in several injury conditions, including oxygen glucose deprivation (Yin et al., 2010) and oxidative injury (Martens et al., 2012; Xu et al., 2011). In our previous work we described that progranulin is up-regulated by hypoxia in neuroblastoma cell lines suggesting that it could exert a protective role in the brain against hypoxic stress, one of the main risk factors involved in FTLD pathogenesis (Piscopo et al., 2010).

It has been hypothesized that ischemia/hypoxia is involved in the pathogenesis of several neurodegenerative diseases (Gerst et al., 1999; Martin et al., 2001). In fact, the CNS is particularly susceptible to changes in local O_2 levels, which can affect neuronal activity (Peña and Ramirez, 2005), and promote the development of disorders, including dementia (Bazan et al., 2002). Several studies have documented that periods of chronic hypoxia predispose individuals to the development of dementia (Peers et al., 2009). Our previous study showed that perinatal hypoxia triggers an early and transient oxidative stress in rat brain, followed by a biphasic regulation of several molecules involved in anti-oxidant defenses, neuroprotection and brain development. The early up-regulation of such molecules is likely to represent an adaptive response of the brain to counteract the consequences of the hypoxic insult (Piscopo et al., 2008).

1.2 Hypoxia and miRNA

MicroRNAs (miRNAs) are single-stranded 21-22 nucleotide small noncoding RNAs, constituting the most abundant class of small RNAs in animals. They have an important role in post-transcriptional regulation of gene expression, by base pairing with target messenger RNAs (mRNAs) (Bartel, 2004). miRNAs can act by translational repression or by cleavage in a sequence-specific manner, depending on the degree of sequence complementarity with their target mRNA (Pillai et al., 2007).

Several studies showed an involvement of miRNAs in different biological processes, such as proliferation, cell differentiation and apoptosis. Moreover, miRNAs have been linked to neurodegenerative diseases (Grasso et al., 2014; Grasso* et al., 2015). A specific family of miRNAs, called hypoxamirs, is altered when cells are in low-oxygen conditions, causing a dysregulation of pathways involved in oncogenesis, angiogenesis, apoptosis (Kulshreshtha et al., 2008, 2007; Gorospe et al., 2011; Nallamshetty et al., 2013) and in different disorders of the central nervous system including stroke, head trauma, neoplasia and neurodegenerative diseases (Acker and Acker, 2004). Although cancer and neurodegeneration are very different pathologies characterized by opposing cell fate, they share an altered oxygen homeostasis and common hypoxia signaling. On one side, cancer cells use the hypoxic response to support their growth, while on the other side this protective mechanism is destroyed in neurodegenerative diseases (Quaegebeur and Carmeliet, 2010).

Hypoxia-activated pathways regulating hypoxamirs are under investigation, but the involvement of Hypoxia-Inducible Factor (HIF) is well known in the regulation of transcriptional changes during hypoxic stress (Semenza, 2012). Several other transcription factors such as NF-κB, PU.1, and p53 also have important roles in the presence of low-oxygen conditions (Cummins and Taylor, 2005), therefore it can be hypothesized that in the regulation of hypoxamirs expression following hypoxia both HIF-dependent and HIF-independent (Nallamshetty et al., 2013) pathways intervene.

1.3 PGRN and miRNAs

FTLD shows several distinct clinical presentations differing not only among mutations, but also within a single mutation and even within individual families, suggesting the involvement of post-transcriptional regulation mechanisms. Recent findings suggest that *GRN* is under the control of miRNAs. In FTLD patients with a common genetic variant of *GRN* (rs5848) the reinforcement of a miR-659-3p binding site due to the presence of T-variant has been suggested to be a risk factor (Rademakers et al., 2008). miR-29b has a role in the regulation of *GRN* expression levels in stable cell line (hPGRN-3T3) expressing full-length human *GRN* cDNA (including the 3′UTR) (Jiao et al., 2010). *GRN* expression is also under post-transcriptional control of miR-107, a member of a miRNA group including miR-15, miR-16, miR-103, miR-195, miR-424, miR-497, miR-503, and miR-646, with implications for brain disorders (Wang et al., 2010). Moreover, a profile of miRNA expression in the frontal cortex of a population of FTLD-TDP patients with *GRN* mutations has been identified (Kocerha et al., 2011). More recently, it was demonstrated that decreased levels of miR-132/212 lead to transmembrane protein 106B (TMEM106B) up-regulation and, consequently, a perturbation of PGRN pathways and increased risk to develop FTLD-TDP (Chen-Plotkin et al., 2012).

The aim of the present study is to examine the role of miR-659-3p in progranulin post-transcriptional regulation and the involvement of this miRNA in *GRN* up-regulation mediated by hypoxic/ischemic insults comparing different in vitro and in vivo models, to verify the hypothesis of a mechanistic link between hypoxic stress and miRNA-mediated modulation of the *GRN* gene.

2. Materials and methods

2.1 miRNA target sites prediction

The prediction of miRNA target sites was performed using the algorithm Targetscan (http://www.targetscan.org/ Version 6.2).

2.2 HeLa cell cultures and transfection

The human cervical carcinoma HeLa cell line was grown in DMEM medium (Gibco®, Life Technologies) supplemented with 2 mM L-Glutamine, Penicillin/Streptomycin and 10% Fetal Bovine Serum (FBS). Cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cells were seeded on 24-well dishes (Luciferase Assay) and 6-well dishes (Western Blotting and ELISA assay) and transfected at 80% confluence with Lipofectamine LTX and Plus reagent (Life Technologies).

2.3 Generation of *GRN* 3'UTR reporter construct and miRNAs-overexpressing plasmids

In order to generate pGLO-*GRN*-3'UTR reporter construct, *GRN* 3'UTR forward and reverse primers (Table 1) were used to amplify human *GRN* 3'UTR from human genomic DNA (#G1471 Promega) and then cloned into pGLO Vector (Promega). This vector is based on Promega Dual-Luciferase technology, with firefly luciferase (luc2) used as primary reporter and Renilla luciferase (hRluc-neo) as a control reporter for normalization.

The miRNA constitutive-expression cassettes for miR-659 and miR-181a (negative control) were generated by PCR amplification of human genomic DNA (#G1471 Promega) and primers reported in Table 1. The genomic fragment containing the pre-miRNA was cloned in the BglII and XhoI sites of pSiUx plasmid (Denti et al., 2004).

2.4 Luciferase Assay

75.000 cells per well were seeded in 24-well dishes and transfected at 80% confluence using Lipofectamine LTX and Plus reagent (Life Technologies) with 15 ng of the pGLO-*GRN*-3′UTR and 435 ng of miRNA-overexpressing plasmids. The pGLO vector is designed to analyze miRNA activity by the insertion of miRNA target sites downstream of the firefly luciferase gene (luc2). miRNA binding to the target sequence will produce a reduced firefly luciferase expression. 24 and 48h after transfection cells were lysed with Luciferase Assay Reagent (Promega), and Renilla and Firefly luciferase activity were measured using Dual-Glo Luciferase Assay System (Promega) in the Infinite® M200 (Tecan) plate reader.

2.5 Western blotting and ELISA assay

HeLa cells were seeded in a 6-well plate and transfected using Lipofectamine LTX and Plus reagent (Life Technologies) with 2.2 μg of miRNA-overexpressing plasmids. After 48h, proteins were extracted by Radioimmunoprecipitation Assay Buffer (RIPA) supplemented with a protease inhibitor cocktail (Sigma-Aldrich) and analyzed by Western Blotting and ELISA assays.

20 μg of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred on nitrocellulose membrane by using the iBlot® Dry Blotting System (Life Technologies) at 20V for 7 minutes. Blots were first blocked with 5% non-fat powdered milk in TBS/Tween 0.1%, then probed overnight at 4 °C with mouse monoclonal anti-GRN (Abcam® no. ab55167, 1:500), and rabbit polyclonal anti-HPRT (FL-218) (Santa Cruz Biotechnology® no. sc-20975, 1:500). Membranes were washed, incubated with IRDye® 680LT Donkey anti-Mouse IgG and IRDye® 800CW Goat Anti-Rabbit IgG secondary antibodies (LI-COR Biosciences, 1:10.000). Membranes were scanned with the LI-COR Odyssey Infrared Imaging System according to the manufacturer's instructions. Densitometric analysis was performed using ImageJ.

1.5 μg of proteins were used to perform ELISA assay (Adipogen). Standards and samples were pipetted into the wells of a 96-well plate for binding to the pre-coated polyclonal antibody specific

for PGRN. After washing to remove unbound samples, PGRN was recognized by the addition of a biotinylated polyclonal antibody. After removal of excess biotinylated antibody, streptavidin labeled with HRP was added. After a final wash, peroxidase activity was quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB) and the intensity of the color reaction, directly proportional to the concentration of GRN in the samples, was measured at 450 nm.

2.6 miR-CATCH technique

A miRNA:target pull down protocol was performed to isolate *GRN* mRNA with its bound miRNAs using a technique described in a recent publication (Hassan et al., 2013) with modifications. Briefly, 5' biotin-modified DNA oligonucleotides (5'-TCTTCAAGGCTTGTGGGTCTGGCAGG-3') (Table 1) complementary to *GRN* mRNA were designed using Mfold (http://mfold.rna.albany.edu/?q=mfold) and synthesized. Three T75 flasks of SK-N-BE cells at full confluence were harvested, cross-linked with 1% formaldehyde for 15 minutes at room temperature after which the reaction was stopped with 0.2 M glycine. After a triple wash with ice cold PBS, the cells were mechanically disrupted in FA-lysis buffer (50mM HEPES pH 7.5, 140mM NaCl, 1mM EDTA, 1% Triton, 0.1 % sodium deoxycholate). Lysates were incubated with oligonucleotide-conjugated streptavidin-coated magnetic beads (Dynabeads MyOne Streptavidin C1) in 50% v/v hybridization buffer (2X Tris-EDTA, 1 M LiCl) for 90 minutes at 37°C. A separate sample was incubated similarly, but with beads conjugated with a non-targeting scrambled control oligonucleotide (Table 1). After three 37°C washes with Wash buffer A (1X Tris-EDTA, 0.15 M LiCl, 0.5% sodium dodecyl sulphate) and two washes with Wash buffer B (1X Tris-EDTA, 0.15 M LiCl), the mRNA:microRNAs complexes were eluted from the magnetic beads and the samples were incubated at 70°C for 45 minutes to reverse the crosslinks. Quantative real time PCR (RT-PCR) was performed to check for the presence of the *GRN* mRNA and exclude possible contamination of other mRNAs and Taqman miRNA assay on miR-659-3p was used to analyze the presence and abundance of this miRNA compared to the scramble non-specific control.

Table 1. Primer sequences

OLIGO	SEQUENCE
Primer Forward *GRN* 3'UTR	5'-AAATCTAGAGGGACAGTACTGAAG-3'
Primer Reverse *GRN* 3'UTR	5'- ATCTAGAGAAAGTGTACAAACTTTATTG-3'
Primer Forward miR-659	5'-ACTGCTCGAGCACTGTCATTATTTTCTCAC-3'
Primer Reverse miR-659	5'-ACTGAGATCTGCGTTCTTGTTTTGTGTTTC-3'
Primer Forward miR-181a	5'-ACTGAGATCTACCATTCAAAGACATTTTCT-3'
Primer Reverse miR-181a	5'-ACTGCTCGAGCTCCTTACCTTGTTGAAATG-3'
Oligo Capture GRN	5'-TCTTCAAGGCTTGTGGGTCTGGCAGG-3'
Scrambled	5'-ATATATTAGATTGCGTATAATTAGG-3'
Primer Forward GRN	5'-TTCTGGACAAATGGCCCAC-3'
Primer Reverse GRN	5'-ACCCACGGAGTTGTTACCTG-3'
Primer Forward GAPDH	5'-TCTCCTCTGACTTCAACAGC-3'
Primer Reverse GAPDH	5'-CGTTGTCATACCAGGAAATGA-3'

2.7 SK-N-BE cell cultures and treatments

Human neuroblastoma cell lines SK-N-BE were cultivated in RPMI-1640 medium (Euroclone). All growth media were supplemented with 10% heat-inactivated (v/v) Foetal Bovine Serum (FBS), 5 mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. For experiments, cells were seeded on 60 mm plastic culture dishes at a density of $1x10^4/cm^2$ and grown to 80% confluence, at which point, the medium was changed. The general morphology of cell monolayers, before and after treatments, was monitored by light microscopy. Cell proliferation was estimated by counting the total number of cells in each dish with use of a hematocytometer. Cell viability was determined by Trypan blue dye exclusion test (Sigma-Aldrich). We also evaluated the number of cells that detached from the substrate and were found to be freely floating in cell medium.

For stimulating hypoxia, we used a hypoxic/anaerobic chamber (BBL™ GasPak™, USA). The system was set up at 37°C in 5% CO_2, 95% N_2. Cells were transferred into the humidified chamber and incubated with the appropriate media for up to 24 h then, lysed for RNA isolation (below). Control cells were maintained in the incubator under normoxic conditions.

2.8 Animal model

Wistar rats were purchased from Charles River (Calco, Italy) and kept in the Animal Facility of the Istituto Superiore di Sanità in an air-conditioned room at 21±1°C and 60 ± 10 % relative humidity, with a white/red light cycle (white light on from 8.30 to 20.30). One week after the arrival of the rats, breeding pairs were formed, and after 48 hours, females were individually housed until the 22nd day of gestation, when the caesarean section was carried out.

The experimental protocol was conducted according to the EC guidelines (EU Directive 26/2010) and the Italian legislation, and under permission of the Italian Ministry of Health. Asphyxia was induced in pups delivered by caesarean section on pregnant Wistar rats, as described by Bjelke et al. (1991). Time-mated female rats within the last day of gestation were decapitated and the entire uterus was quickly removed. Use of anesthetic in the dam was avoided because maternal anesthesia affects the degree of CNS hypoxia experienced by the neonate. Immediately after hysterectomy, four pups were delivered to be considered caesarean-delivered (0 min asphyxia) controls, then the uterus horns, still containing the fetuses were placed in a saline bath maintained at 37°C. Pups from the same uterus were then removed after 20 min and stimulated to breath by gently touching them around the oral and genital areas for approximately ten minutes. Fetuses were assigned randomly to the different asphyxia condition. Pups were then left to recover for approximately 1 h in a humidity- and temperature-controlled incubator at 30±1°C (Elmed Ginevri 0GB 1000, Rome, Italy) until fostered by surrogate mothers that had given birth to healthy litters within 24 h. One male and one female from each asphyxia condition (0 min, 20 min) were assigned to a foster dam, (8 pups/litter). Pups were sacrificed for molecular and biochemical studies at three time points: post-natal day (pnd) 1, 4 or 11. At least 4 animals per groups were used at each time points.

2.9 Quantitative RT-PCR

Total RNA was extracted from cell and brain samples using the Invisorb SpinCell RNA kit (Invitek). For progranulin expression cDNA was synthesized by retrotranscription using

SuperScript III first-strand cDNA synthesis kit (Invitrogen Inc., Carlsbad, CA) with random primers, according to the manufacturer's protocol. Reverse transcription quantitative real-time PCR (RT-qPCR) was performed using a specific TaqMan Gene expression assay (Applied Biosystems); 18S rRNA was chosen as a reference gene. The parameters for PCR amplification were: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. PCR was performed in triplicate for each sample; 18S rRNA was chosen as reference gene.

Total RNA was extracted from SK-N-BE cells using TRIzol reagent (Invitrogen), according to the manufacturer's instruction. For the quantification of *GRN* transcripts, cDNA was synthesized by retrotranscription using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) with oligo(dT) primers, according to the manufacturer's protocol. Real-Time PCR (RT-qPCR) was performed using Kapa SYBR fast qPCR master mix (Kapa Biosystem) and specific primer reported in Table 1. The expression of *GRN* mRNA was normalized by *GAPDH* reference.

TaqMan MicroRNA Reverse Transcription kit (Life Technologies) was used for miRNAs quantification in SK-N-BE and Hela cells. Starting from total RNA (10 ng), we converted miRNA to cDNA using reverse transcriptase and miRNA-specific stem–loop primers: hsa-miR-659-3p (001514), hsa-miR-181a-5p (000480), and endogenous controls RNU44 (001094) and RNU48 (001006). Rat miR-659-3p was quantified using TaqMan MicroRNA Reverse Transcription kit for hsa-miR-659-3p (001514) and endogenous controls RNU44 (001094) and RNU48 (001006). The PCR reaction (20 µl), containing 1.33 µl of cDNA, 10 µl of TaqMan 2X Universal PCR Master Mix, 1 µl of TaqMan MicroRNA Assay (20X) containing probes specific for the miRNAs of interest, was incubated at 95°C for 10 min, and then at 95°C for 15 s and 60°C for 60 s for 40 cycles. The relative expression of mRNAs and miRNAs was calculated by using the comparative Ct method. Data were expressed as fold-change relative to the mean of endogenous controls.

2.10 Sequencing

HeLa and SK-N-BE 3'UTR region of *GRN* gene was sequenced with specific primers (Table 1) by using the BMR-Genomics sequencing service (BMR Genomics, University of Padova, Padova, Italy, http://www.bmr-genomics.it).

2.11 Statistical analysis

Data are expressed as mean±SEM. Comparisons among groups were made using Student's t-test with significance set at $p<0.05$. Spearman's rank correlation coefficient was calculated to assess association between levels of mRNA and miRNA.

3. Results

3.1 Bioinformatic prediction of miR-659-3p target site in *GRN* 3'UTR

With the aim of identifying miRNAs regulating *GRN*, its 3'UTR was analysed using the algorithm Targetscan. Among several miRNA binding sites, Targetscan predicted the presence of a miR-659-3p binding site in the 3'UTR of *GRN*. It has been previously observed that in FTLD patients with a common genetic variant of *GRN* (rs5848) there is a nucleotide substitution from C- to T-allele in the miR-659-3p binding site that should strengthen the binding of miR-659-3p to the *GRN* mRNA (Rademakers et al., 2008). Rademakers and colleagues described that depending on the presence of the C-allele or the T-allele, the positioning of miR-659-3p with respect to the miRNA binding site in *GRN* was expected to shift, resulting in the formation of three additional base-pairs at the 5' end of the miRNA when the risk T-allele of rs5848 was present. The stronger binding of miR-659-3p to the *GRN* mRNA containing the T-allele was expected to result in a more efficient inhibition of PGRN translation leading to reduced PGRN expression levels.

However, luciferase assays in the work by Rademakers and colleagues suggest that miR-659-3p only binds the transcript of the T allele. To investigate the binding of miR-659-3p to either the C

allele or T allele *GRN* transcript and to calculate the hybridization minimum free energy in both cases we analysed both sequences with RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid) (Rehmsmeier et al., 2004). The *in silico* analysis confirmed that miR-659-3p can bind the *GRN* mRNA C allele with a DeltaG of -23.5 kcal/mol (Fig.1A) although the binding is stronger to the T allele transcript (DeltaG = -25.6 kcal/mol) (Fig. 1B).

A

```
mfe: -23.5 kcal/mol

target 5'    A  A      CCU     A  3'
              GC CCUCCC   AACCA
              UG GGAGGG   UUGGU
miRNA  3' CCCC         AC      UC 5'
```

B

```
mfe: -25.6 kcal/mol

target 5'    C   U            A  3'
              ACC CUCCCU AACCA
              UGG GAGGGA UUGGU
miRNA  3' CCCC          C     UC 5'
```

Figure 1. Thermodynamics of miR-659-3p binding to *GRN* transcript.
Comparison between base-pairing in the presence of wild-type C-allele (**A**) or risk T-allele (**B**). The presence of the risk T-allele results in a stronger binding (DeltaG = -25.6 kcal/mol).
MFE, minimum free energy change (ΔG).

3.2 Functional assays to validate the predicted miR-659-3p target site

To verify that miR-659-3p binds the C-allele *GRN* 3‘UTR, HeLa cells were co-transfected with a miR-659-overexpressing plasmid (pSiUx-miR-659) and pGLO-*GRN*-3’UTR (a pGLO plasmid containing the C-allele *GRN* 3’-UTR cloned downstream of the firefly luciferase gene). A plasmid overexpressing miR-181a (pSiUx-miR-181a) was used as negative control, since no binding site for miR-181a-5p was predicted on *GRN* 3’-UTR. The levels of miR-659-3p, were significantly increased at 24 hours and 48 hours, as measured by qRT-PCR on mature miRNAs (Fig. 2). The levels of miR-181a-5p, already present in HeLa cells, were also increased at 24 hours and 48 hours albeit by smaller amounts.

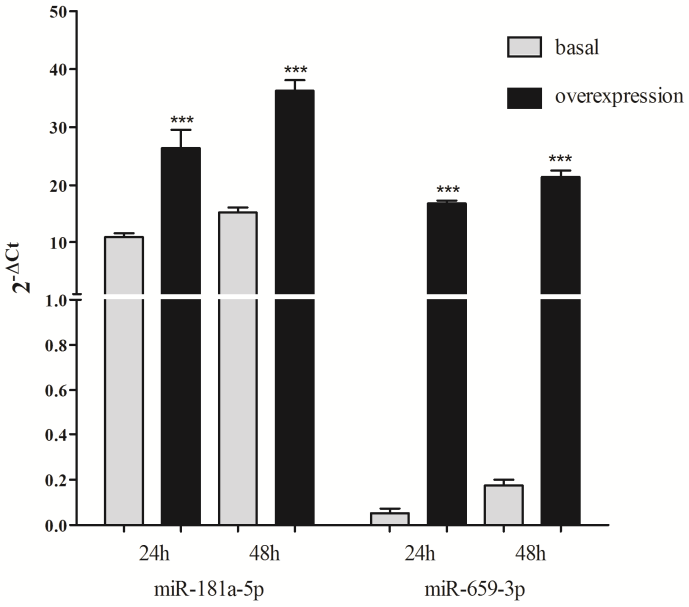

Figure 2. Upon transfection of pSiUx-miR-659 and pSiUx-miR-181a, miRNAs are overexpressed. expression levels of miRNAs measured by qRT-PCR after 24h and 48h from transfection. Mean ± SEM of two biological replicates is shown (N=6, *** $p < 0.001$ vs. miRNAs basal expression levels).

The co-transfection of HeLa cells with these constructs showed that miR-659-3p but not miR-181a-5p overexpression led to a significant decrease in luciferase expression from the pGLO-*GRN*-3'UTR reporter compared to cells transfected with pSiUx-empty (Fig. 3). Moreover, miR-659-3p overexpression induced a reduction of luciferase activity at 24h (Fig. 3A) and 48h (Fig. 3B), compared to the luciferase activity measured in the same cells transfected with miR-181a-overespressing plasmid. These results suggest a direct binding of miR-659-3p on the C-allele *GRN* 3'-UTR.

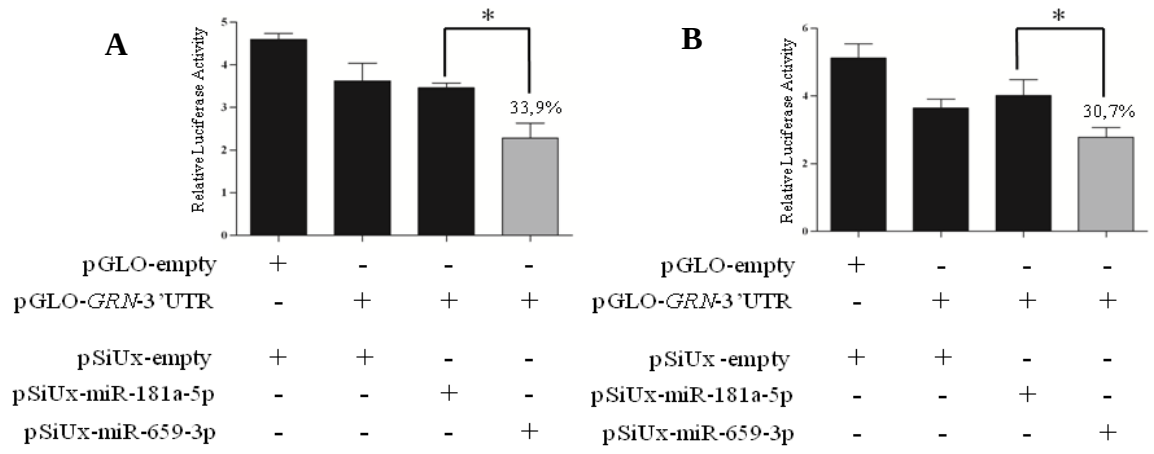

Figure 3. Luciferase assays confirm the interaction between miR-659-3p and the C-allele *GRN* 3'UTR.
GRN 3'-UTR was cloned downstream of the firefly luciferase gene in pGLO vector and co-transfected with miR-659 and miR-181a-overexpressing plasmids in HeLa cells. Luciferase activity was assessed 24h (A) and 48h (B) after transfection (N=3; * $p < 0.05$).

In order to characterize the HeLa cell model system with respect to SNP rs5848, *GRN* 3'UTR region of HeLa cells was sequenced. In Fig. 4A is shown that this cell line presented a CC genotype. To further validate the effect of miR-659-3p on the expression of the endogenous PGRN expression, HeLa cells were transfected with either pSiUx-miR-659 or pSiUx-miR-181a. 48h after transfection, a ~25% reduction of C-allele PGRN measured by ELISA assay was observed in cells overexpressing miR-659-3p, compared to cells transfected with the negative control pSiUx-miR-181a (Fig. 4B). A similar result was shown by Western Blot analysis, in which the transfection of miR-659-overexpressing plasmid caused a ~15% reduction of HeLa endogenous C-allele PGRN (Fig. 4C).

HeLa cells were chosen for these experiments since they have a low basal expression of miR-659-3p (Fig. 1).

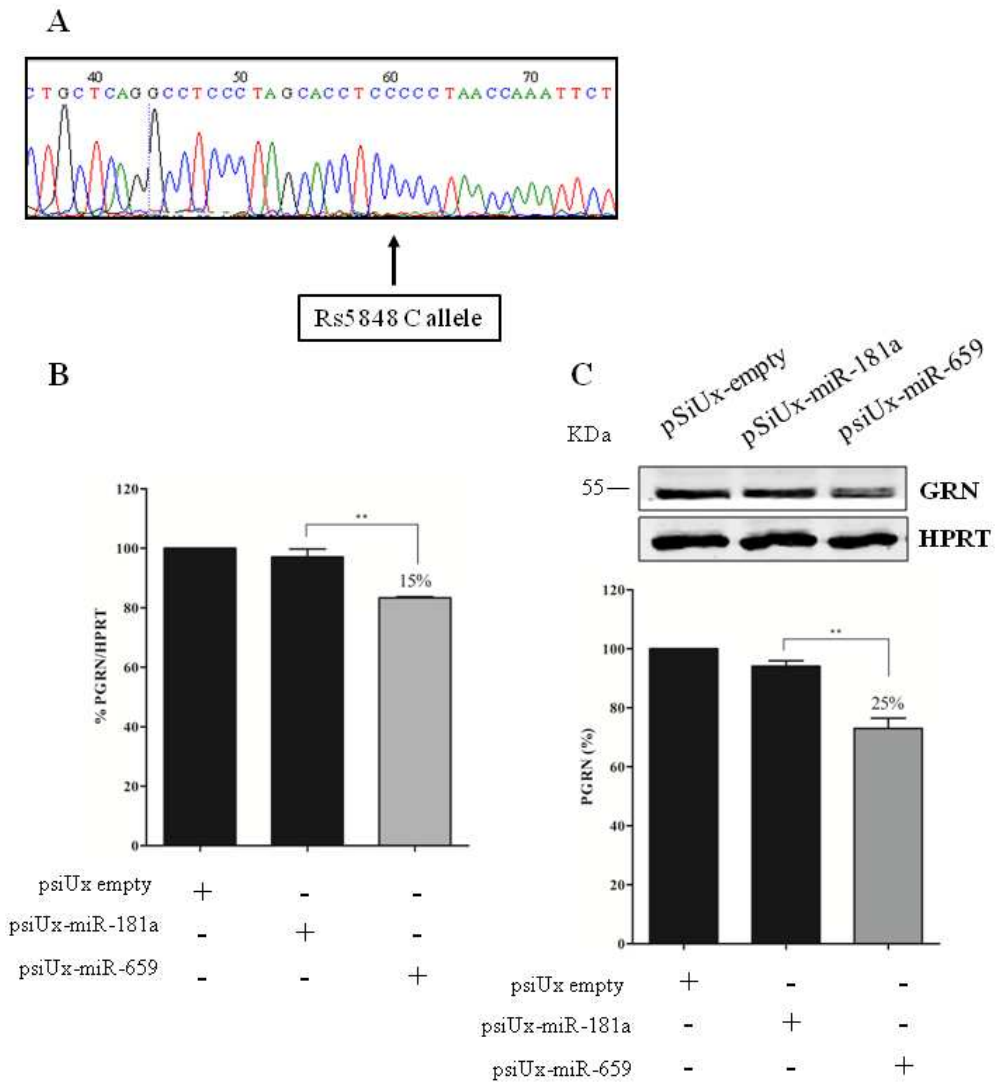

Figure 4. Inverse correlation between PGRN expression levels and miR-659-3p in HeLa cells.

HeLa 3'UTR sequencing showing a CC genotype (A). ELISA assay (B) for PGRN quantification in protein extracts from HeLa cells (N=4; ** $p < 0.01$).
Western blot analysis (C) for PGRN detection in protein extracts from HeLa cells (N=3; ** $p < 0.01$). Western Blot image is representative of three biological replicates. Expression of Western Blot was normalized on HPRT expression.

To confirm the physical interaction between *GRN* mRNA and miR-659-3p a pull down protocol was performed using a technique we described in a recent publication (Hassan et al., 2013). For the following experiments, we chose SK-N-BE, a human neuron-like cell culture representing a more similar environment to FTLD pathology. Moreover, this neuroblastoma cell line was used in our previous work describing the up-regulation of PGRN by hypoxia (Piscopo et al., 2010).

With the aim of characterizing SK-N-BE cell model system with respect to SNP rs5848, as for HeLa cells, *GRN* 3'UTR region was sequenced in SK-N-BE cell line showing a TC genotype (Fig. 5A).

qRT-PCR showed a 23,73-fold enrichment for *GRN* mRNA using a *GRN* mRNA-specific capture oligonucleotide compared to a scramble oligonucleotide used as negative control (Fig. 5B). In parallel, Taqman assay showed a 311,5-fold enrichment of miR-659-3p in samples captured with *GRN* mRNA-specific oligonucleotide (Fig. 5C).

Taken together, these results suggest that miR-659-3p can affect PGRN expression in HeLa and SK-N-BE cells.

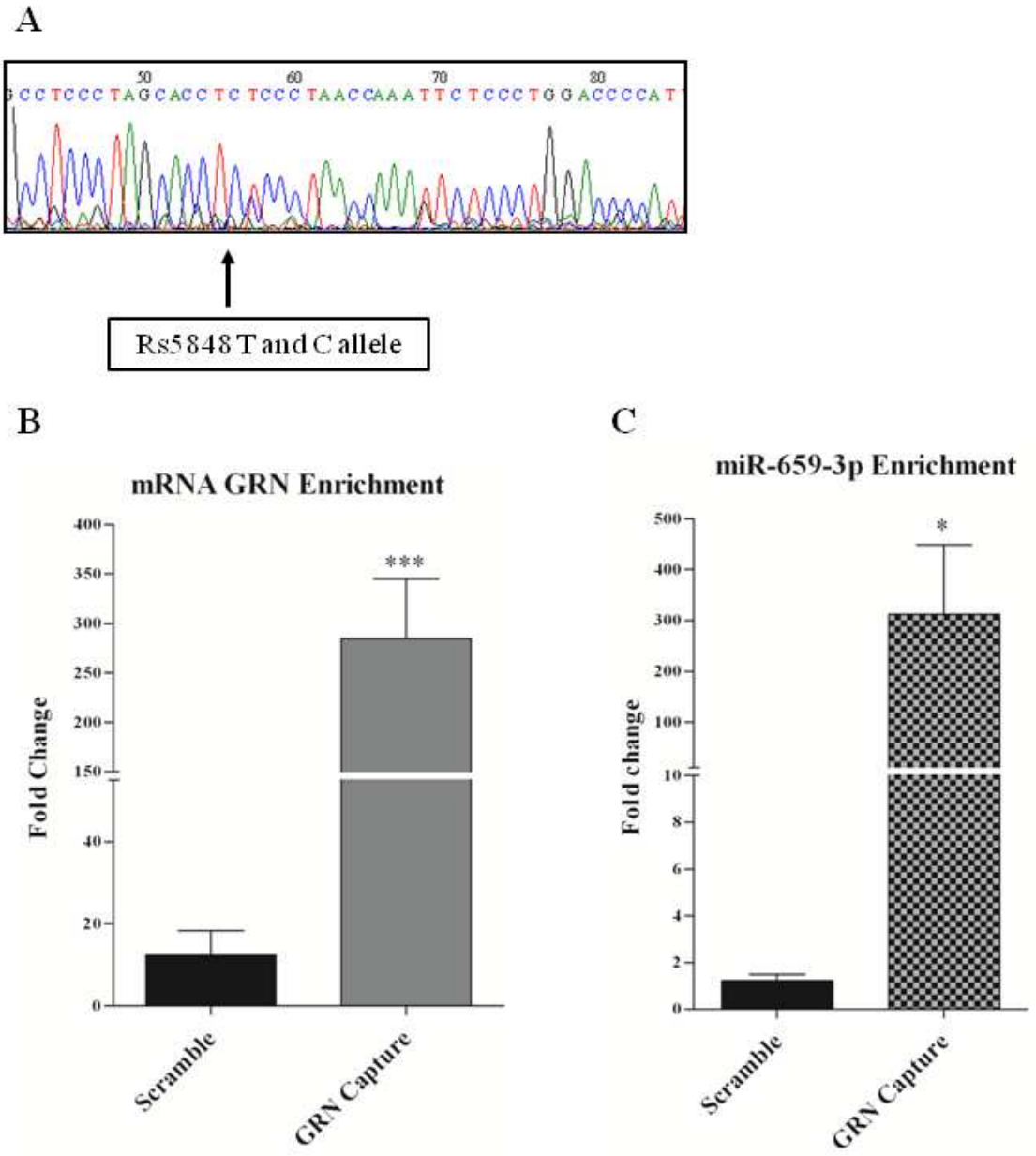

Figure 5. mRNA:miRNA isolation technique for *GRN* mRNA from SK-N-BE cells.

SK-N-BE 3'UTR sequencing showing a TC genotype (A). A capture anti-sense DNA oligonucleotide with a biotin modification at the 5' end was designed to pull-down *GRN* mRNA. qRT-PCR showed enrichment of *GRN* mRNA (B) and of miR-659-3p (C) in samples captured with *GRN* mRNA-specific oligonucleotide compared to a scramble oligonucleotide used as negative control. mRNA expression was quantified with the $2^{-\Delta\Delta Ct}$ method using GAPDH as housekeeping gene; miRNA expression was quantified using the $2^{-\Delta Ct}$ method.
Mean ± SEM of three biological replicates is shown (N=9, * $p < 0.05$; *** $p < 0.001$ vs. scramble non specific control).

3.3 miR-659-3p levels are correlated with *GRN* mRNA expression in SK-N-BE

In order to test the effect of hypoxia on the miR-659-3p-mediated regulation of PGRN, the levels of PGRN transcript and protein and of miR-659-3p were analysed in SK-N-BE under normoxic and hypoxic conditions. As a marker of hypoxia, the expression of glucose transporter GLUT1, which is regulated under hypoxic condition (Fisk et al., 2007), was measured. As shown in Fig. 6, GLUT1 levels were up-regulated in response to hypoxia, with a dramatical increase at 24 h of incubation. Moreover, 24 h of hypoxic treatment was not cytotoxic for the cells, at least under the assay conditions we have used here. In fact, the number of cells treated with hypoxia did not differ by more than 5% from the number of control cells, without any decrease in cell viability (96±2 %) (data not shown). In the same samples, *GRN* mRNA was increased 2.44±0.15-fold (Fig. 6).

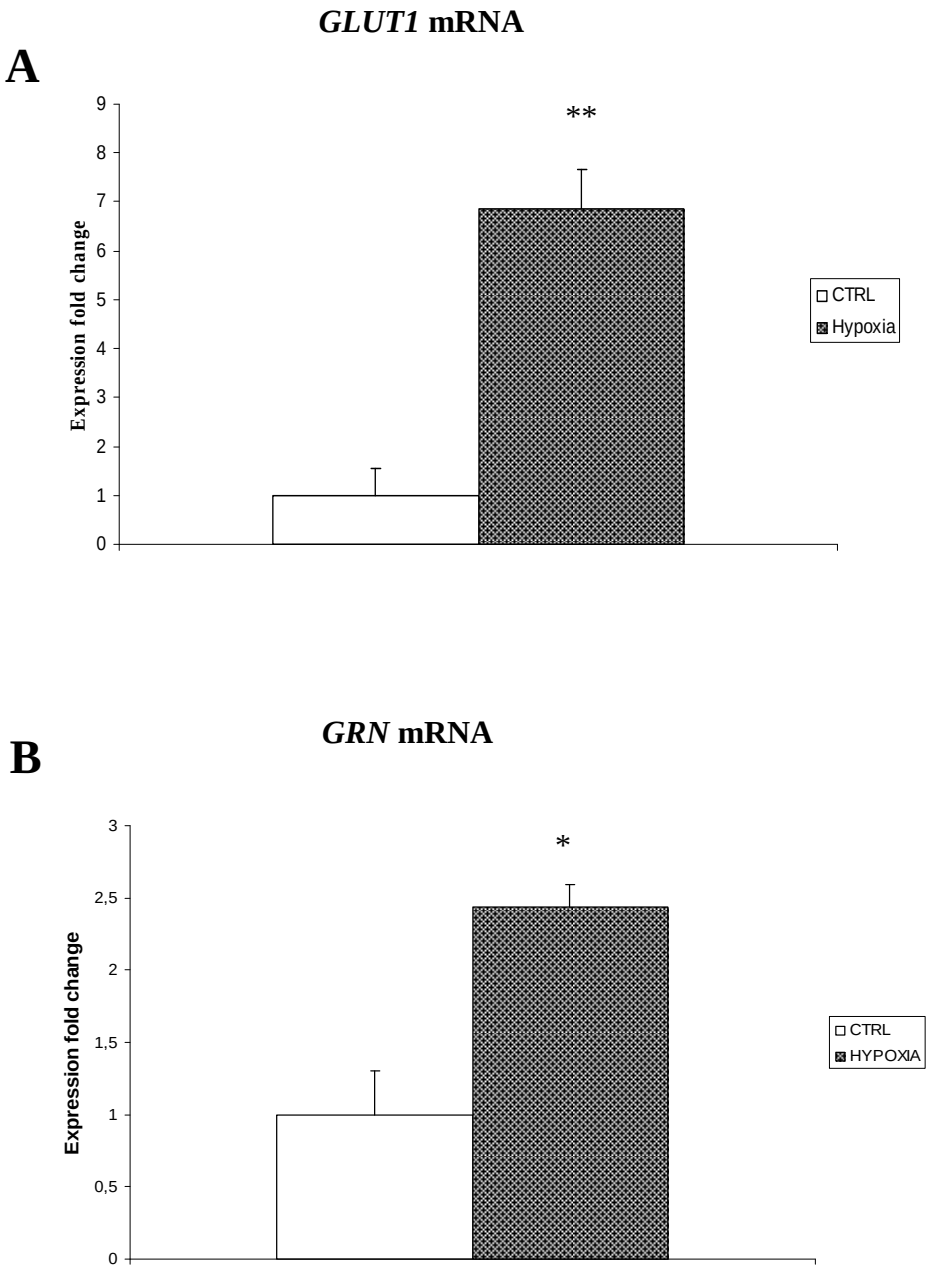

Figure 6. Twenty-four hours of hypoxia treatment affect *GLUT*-1 and *GRN* gene expression in SK-N-BE cell lines **(A, B).** The histograms show mRNA levels of *GLUT-1* **(A)** and *GRN* **(B)** after hypoxia administration. Data, compared to 18S rRNA, are expressed as fold changes relative to the control at each treatment. Mean ± SEM of six experiments is shown.
***P*< 0.01 and **P*< 0.05 vs. untreated normoxic cells.

The levels of miR-659-3p were also analysed and a possible correlation between miR-659-3p expression and *GRN* mRNA was investigated. We observed a negative correlation between *GRN* mRNA and miR-659-3p expression levels (Spearman's rank correlation coefficient = -0.96, p = 0.0005) (Fig. 7).

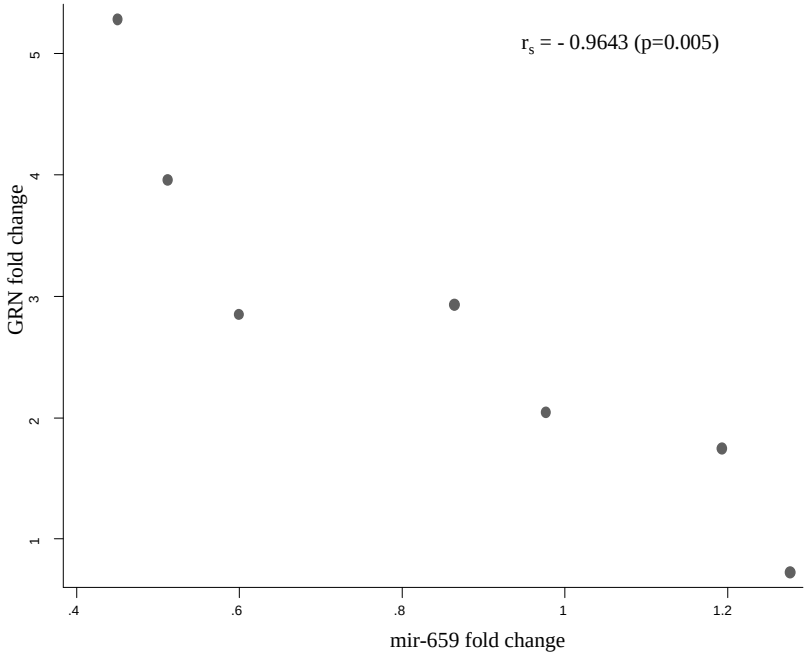

Figure 7. Negative correlation between *GRN* and miR-659-3p expression levels in SK-N-BE.

3.5 GRN increase and miR-659-3p decrease in a rat model of global perinatal asphyxia

In order to evaluate whether the hypoxia-induced miR-659-mediated regulation of PGRN takes place also *in vivo*, *GRN* mRNA and miR-659-3p levels were measured in a rat model of global perinatal asphyxia. We have previously shown that 20 min of global asphyxia are required for inducing significant brain oxidative stress, measured as increased levels of the lipid peroxidation product F2-isoprostane, and alterations in the spontaneous motor behavior (Calamandrei et al., 2004). Shorter times of asphyxia (5, 10 or 15 min) did not cause significant alterations at biochemical and behavioral levels, whereas longer periods of asphyxia (>25 min) were

characterized by low survival rates. Thus, in the present study we adopted two exposure conditions: 0 and 20 min of asphyxia. In these conditions, survival rate was 100% and 95% in the 0 min and 20 min of asphyxia group, respectively; body weight on pnd 11 was lower in pups subjected to 20 min perinatal asphyxia in comparison to control rats (approximately 8% decrease), as previously described (Calamandrei et al., 2004). The qRT-PCR on *GRN* transcripts was performed in rat cortical samples at pnd 1, 4 and 11. 20 min perinatal asphyxia was found to increase *GRN* mRNA levels compared to controls. In particular, hypoxia at birth increased *GRN* levels at pnd1 (2.91±0.21-fold p=0.004) and pnd 4 (increased 3.97±0.49-fold p=0.04) (Fig. 8A). Reciprocally, miR-659-3p levels significantly decrease at pnd 4 (p=0.05) (Fig. 8B).

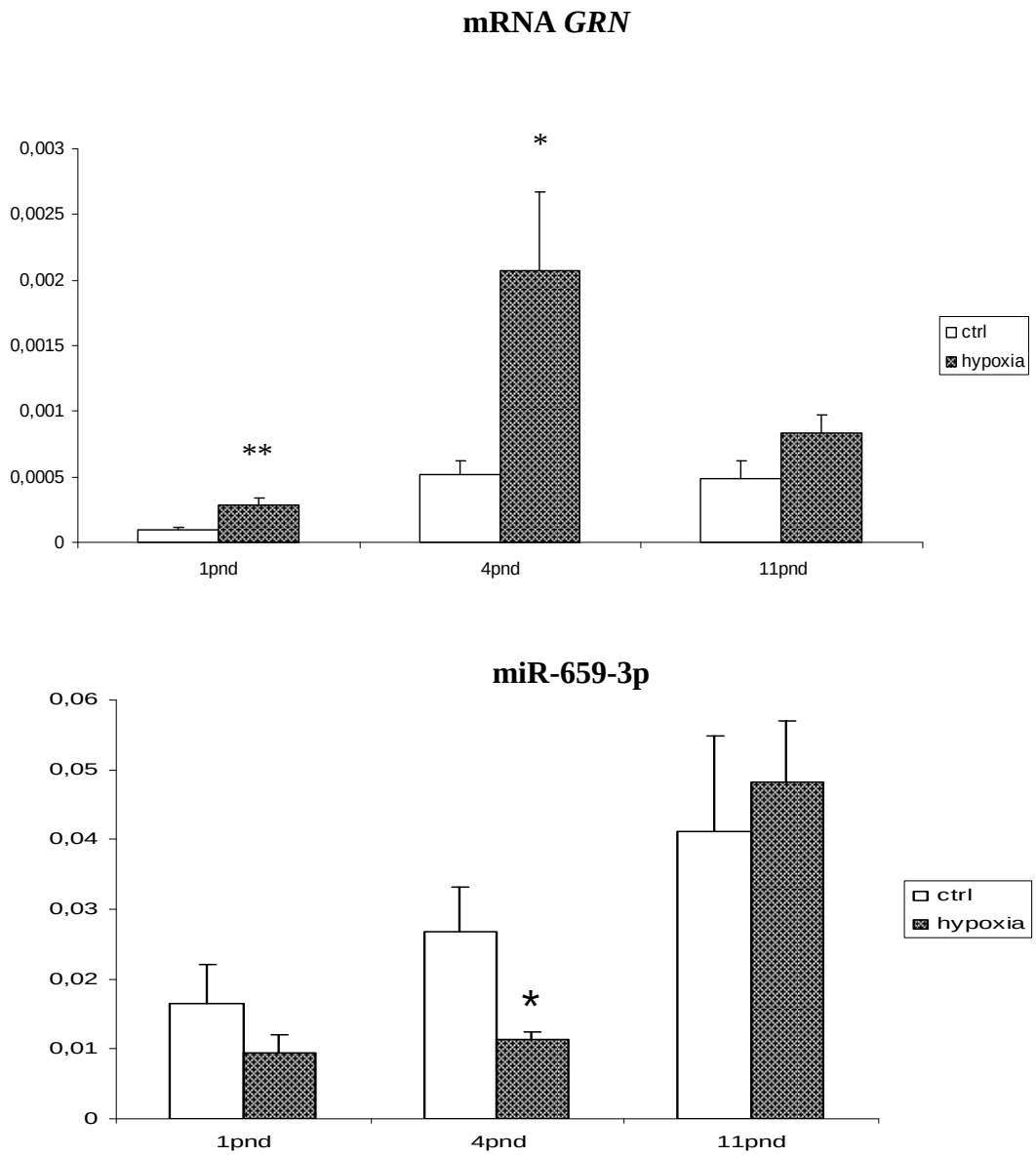

Figure 8. Levels of *GRN* mRNA (A) and miR-659-3p (B) in cortex of control and asphyctic newborn rats at different time points after global perinatal asphyxia. Data, expressed as $2^{-\Delta Ct}$, are means±SEM. N=5

4. Discussion

This study outlines an interaction between miR-659-3p and the *GRN* transcript and indicates a role for this miRNA in the post-transcriptional regulation of *GRN* expression. This claim is supported by multiple findings. First, we found that miR-659-3p interacts directly with the *GRN* 3'-UTR as shown by luciferase assay. Second, we showed by ELISA and Western Blot analysis that miR-659-

3p regulates the expression levels of the endogenous *GRN* in HeLa cells. Finally, we demonstrated the binding between *GRN* mRNA and miR-659-3p in a more physiological condition with a miRNA capture affinity technology (miR-CATCH) in SK-N-BE cells.

Moreover, we found that the interaction between miR-659-3p and the *GRN* transcript participates in the regulatory scheme responsible for the control of *GRN* levels in SK-N-BE cells after hypoxic treatment. In a previous study, we evaluated the expression of progranulin after hypoxic treatment in neuroblastoma cell lines and we found that progranulin is up-regulated by hypoxia suggesting that it could exert a protective role in the brain against hypoxic stress, one of main risk factors involved in dementia (Piscopo et al., 2010). In order to study a possible involvement of miRNAs in hypoxia-mediated up-regulation of *GRN*, we evaluated miR-659-3p levels in SK-N-BE cells after 24 h of hypoxic treatment finding them inversely correlated to *GRN* transcripts. In the miR-659-3p target site on *GRN* 3‘UTR, Rademakers and colleagues described an allelic variant associated to FTLD (Rademakers et al., 2008). They described that homozygous carriers of SNP rs5848 T-allele had a 3.2-fold increased risk to develop FTLD compared with homozygous C-allele carriers. We analysed *GRN* 3’UTR region in our cell lines and found that HeLa cells have a CC- and SK-N-BE cells a CT-genotype. Therefore, we observed a translational repression (shown by Luciferase, Western Blot and ELISA assays) and a physical interaction (using miR-CATCH) of wild-type C-allele with miR-659-3p. In Rademakers work, using a different cell-based system and different conditions, miR-659-3p seems to bind only the risk T-allele, as demonstrated by luciferase assays and *in vivo* studies (Rademakers et al., 2008). Our results add new finding in the regulation of PGRN mediated by miRNAs, showing that also the binding of wild-type C-allele has a role in the post-transcriptional regulation of PGRN.

In order to validate the role of PGRN and miR-659-3p in hypoxic conditions, we extended our experimental work *in vitro* to an animal model of asphyxia. We found that *GRN* mRNA levels were increased at pnd 1 and pnd 4 in cortices of rats subjected to 20 min asphyxia in comparison to

control rats; moreover, although uncharacterized in rat to date, our data indicates that miR-659-3p is decreased at pnd 4 when PGRN reached the highest levels.

These data are strongly consistent with the observation obtained from the SK-N-BE cells after hypoxic treatment. The fact that we observed a significant modulation of miR-659-3p expression only at pnd 4 requires further investigation to fully elucidate the mechanisms regulating PGRN expression. For instance, TDP-43, a DNA and RNA binding protein, was shown to specifically bind *GRN* 3'UTR with a role in the control of *GRN* mRNA stability (Polymenidou et al., 2011; Colombrita et al., 2012). The transmembrane protein TMEM106B has been identified as a potential PGRN regulator considering that TMEM106B up-regulation seems to sequester PGRN in TMEM106B positive late endosomes or lysosomes, and increase intracellular levels of PGRN (Chen-Plotkin et al., 2012). Moreover, a recent work shows that *GRN* mRNA with short and long 5'-UTR is differentially expressed via post-transcriptional and translational repression (Capell et al., 2014).

Taken together these results suggest a possible involvement of miR-659-3p in *GRN* up-regulation mediated by hypoxic/ischemic insults and confirm the importance to study the regulation mechanism of *GRN* expression after hypoxic insult in order to understand its role in dementia.

It has been demonstrated that chronic hypoxia causes a deficiency of Dicer (DICER1) expression and activity, with a resulting deregulation of miRNAs biogenesis (Ho et al., 2012). The downregulation of miR-659-3p after hypoxia observed in our samples could indeed be due to a down-regulation of Dicer expression as shown in Ho and colleagues work (Ho et al, 2012). However, we believe that the effect we observed is not due to a widespread down-regulation, since, after hypoxic treatment, together with a miR-659-3p decrease, we observe other miRNAs, which are up-regulated or non-responsive to hypoxia (data not shown). According to this observation, Caruso and coworkers (Caruso et al., 2010) showed that, during the onset of pulmonary arterial hypertension (PAH) after hypoxia, there is a reduced Dicer expression leading to miR-22, miR-30,

and let-7f down-regulation and, at the same time, to miR-322 and miR-451 up-regulation in two different PAH rat models.

The alteration of miRNAs in neurodegenerative diseases is probably the sum of different factors, but several evidences showed that hypoxia could have a significant impact. Strategies adopting combined approaches including Chromatin Immunoprecipitation (ChIP) or in vitro processing assay could help to understand the response to hypoxia and the pathways leading to miRNAs deregulation. A recent evidence showed that the processing of pre-miR-139 is blocked by inhibitors induced by Hypoxic Ischemia (HI), resulting in the down-regulation of mature miR-139-5p and a consequent up-regulation of Human Growth and Transformation Dependent Protein (HGTD-P), a proapoptotic protein (Qu et al., 2014). The hypothesis of a similar mechanism involving miR-659-3p processing mediated by hypoxia needs to be investigated in our *in vitro* models in further work.

As final consideration, it should be noted that in neurodegeneration we suppose that the up-regulation of PGRN could be linked to neuroprotection in the case of neurodegenerative disorders but also to a more general protective role. This is supported by our previous work in which we described that progranulin exerts a protective role against hypoxic stress in neuoblastoma cell lines (Piscopo et al., 2010). Therefore, it is shown that progranulin is a neuroprotective growth factor, expressed within motor neurons and promoting neuronal cell survival (Ryan et al., 2009). Moreover, it is also part of a fibroblast stress response and cytoprotection to acidotic stress (Guerra et al., 2007) and, very recently, it has been demonstrated that PGRN protects against hypoxia-induced inflammation in a mouse model of renal ischemia/reperfusion injury (Zhou et al., 2015).

However, elevated levels of PGRN in cancer positively stimulate pathways involved in proliferation, migration and invasiveness. The first evidence in cancer showed a role for PGRN as autocrine growth stimulus for an aggressive murine teratoma (Zhou et al., 1993). Then, it was demonstrated an involvement of GRN mRNA expression levels in breast cancer (Lu and Serrero, 2000; Swamydas et al., 2011), liver cancer (Cheung et al., 2004), squamous esophageal cancer (Chen et al., 2008), glioblastoma (Bandey et al., 2014), and gastrointestinal cancer (Demorrow,

2013). More recently, other works showed also a role for PGRN as a possible diagnostic and prognostic cancer biomarker (Han et al., 2011; Wang et al., 2012; Wei et al., 2015). Thus, neurodegeneration shares with cancer an altered oxygen homeostasis and common hypoxia signaling, and although these two pathologies are entirely distinct and have opposite outcome (cancer leads to cellular growth and neurodegeneration resulting in cell death), a better characterization of altered molecular pathways involving PGRN and response to hypoxia is very important for the development of effective therapies for both diseases.

In conclusion, our results demonstrate the interaction between miR-659-3p and *GRN* transcript and the involvement of miR-659-3p in *GRN* up-regulation mediated by hypoxic/ischemic insults.

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